Genetic analysis and functional characterization of factors affecting BM-derived myogenesis

Thesis

Genetic analysis and functional characterization of factors affecting BM-derived myogenesis

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Abstract

Numerous common and severe human muscle disorders could benefit from stem cell therapy that would allow replacement of degenerating muscle. A recent upsurge of reports, claiming that readily accessible haematopoietic stem cells (HSCs) participate in muscle regeneration, raises hopes for their clinical application. However, the biology of this process and the proof of the ability of HSCs to be reprogrammed remain elusive. Using murine bone marrow (BM) cells, carrying different reporter cassettes controlled by elements of the muscle-specific *Myf5*, *MLC3F* and *MCK* promoters, we demonstrated that haematopoietic cells in co-culture with myoblasts express muscle-specific genes. In spite of this, these cells show limited regeneration of muscle in *Pax7*$^{-/-}$ mice and never give rise to myoblast clones. Correspondingly, the gene expression profiling of haematopoietic CD45^{+}/Sca1^{+} cells revealed partial but extended myogenic reprogramming, despite the absence of key myogenic transcription factors such as Pax7 and MyoD. Indeed, BM transplantation and co-culture experiments, using BM cells from *Pax7*$^{-/-}$ or *MyoD*$^{-/-}$ mice, suggested that neither Pax7 nor MyoD are essential for the participation of haematopoietic cells in muscle regeneration, indicating that the Pax7 pathway is not active in these cells. *In-vitro* experiments with muscle cells from *Pax7*$^{-/-}$*/Myf5*$^{nlacZ/+}$ and Pax7$^{-/-}$/MLC3F-*nlacZ*-E mice confirmed the tight association of Pax7 with the muscle-stem-cell characteristics. Additionally, the *Pax7* ectopic expression in BM cells infused to them the ability to generate myogenic clones and increased their *in-vivo* myogenic potential. Overall, our results demonstrate that BM-derived CD45^{+}/Sca1^{+} cells, while undergoing myogenic specification and differentiation, cannot be considered muscle stem cells, and this latter distinction from the satellite cells is most probably due to the inactivity of the Pax7 pathway.

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1 Introduction

1.1 Stem cell biology: a brief overview.

The multicellular organisms are formed by the long-lasting cell division of one single cell, the zygote. In addition, the cells of most tissues can be damaged or die and therefore must be replaced. This need for continuous cell divisions and cell replacement during development and adult life, respectively, is met by a category of "reserve" cells called stem cells. These cells can self-renew (i.e. generate daughter cells identical to themselves) for long periods and extensively contribute to organ formation and tissue maintenance and regeneration. This unique tissue generation capacity is due to their *in-vivo* ability to proliferate and differentiate in several cell types, in large numbers. They have been isolated both from the embryo and the adult organism, and according to their ability to give one or more differentiated cell types they are categorised in totipotent, pluripotent, multipotent or unipotent (Table 1).

In mammals, the zygote and the early blastomeres, until the eight-cell stage, are totipotent and remain plastic [1]. They undergo cleavage divisions and lack the capacity to self-renew. After the formation of the blastocyst, the embryonic stem (ES) cells appear. These cells had been initially isolated in the early 1980s from the inner cell mass (ICM) of mouse blastocyct [2,3] and later by the human one [4]. ES cells can propagate indefinitely and differentiate inot wide variety of cell types *in vitro*. The injection of both mouse and human ES cells in adult mice results in the formation of teratocarcinomas that contain derivatives of all three primary germ layers (endoderm, mesoderm and ectoderm) [2,4]. In addition, the insertion of mouse ES cells into mouse blastocysts leads to the creation of chimaeric mice that contain donor-derived cells in all the adult tissues. These above-mentioned qualities demonstrate that ES cells can give rise to all cell types and

therefore their pluripotency (Figure 1). Furthermore, they can also generate early haematopoietic precursor cells [5], cardiomyocytes [6] and other adult cell types (Figure 2). Although, this latter characteristic makes them excellent candidates for regenerative medicine, the formation of teratomas after their *in-vivo* transplantation and the ethical issues, concerning their derivation from early human embryos, currently forbid their clinical use.

More recently, adult stem cells have also been identified and represent an alternative source for cell transplantation. Nowadays, we know that they exist in most if not all tissues, including bone marrow (BM), intestine, epidermis, bone and muscle, and as mentioned before they are responsible for the replacement of the terminally differentiated cells. In contrast to ES cells, these cells are already determined and therefore they are generally more restricted to their differentiation potential [7]. Their characteristics differ according to the tissue in which contribute and its renewal needs, but they all act through the creation of an intermediate population of amplifying cells prior to their terminal differentiation (Figure 3).

Cell category	Cell example	Characteristics
Totipotent	Zygote and very early blastomeres	Construct the whole organism by itself.
Pluripotent	Embryonic stem cells (ES)	Give rise to all cell types and contribute to all the tissues of the organism.
Multipotent	Haematopoietic stem cells (HSC)	Give rise to multiple cell types.
Unipotent	Muscle stem cells	Differentiate only in one specific cell type.

Table 1. Stem cell classification.

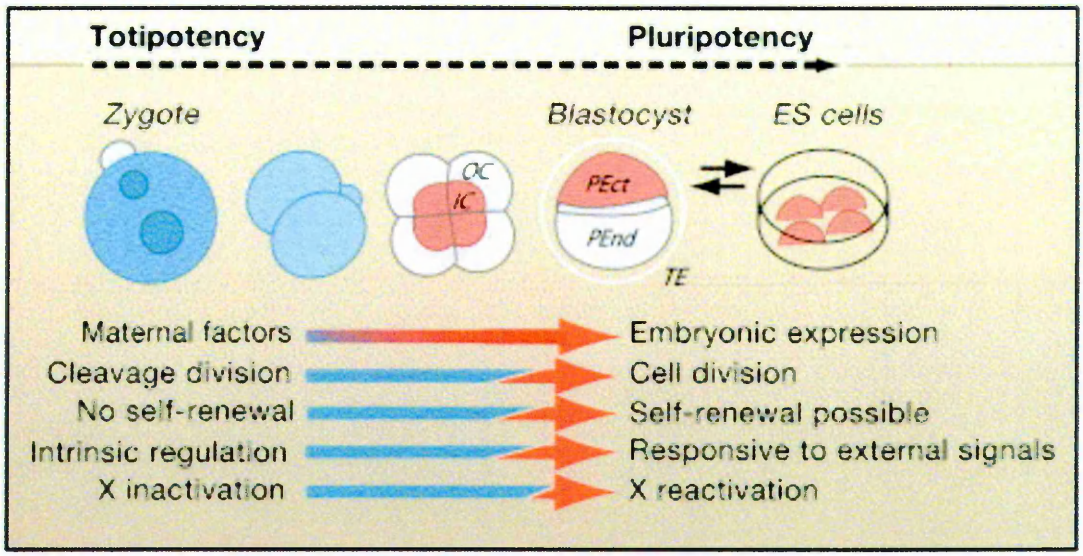

Figure 1. The Transition from Totipotency to Pluripotency[1].

In mammals, the zygote and the first eight cells formed, which are identical to each other, are the only cells that have the potential to create the whole organism (totipotent). This property is lost after the eight-cell stage, however the cells isolated from the inner mass of the blastocyst, called embryonic stem cells, retain their ability to give rise to all three germ layers and therefore to all the tissues of the adult organism (pluripotent).

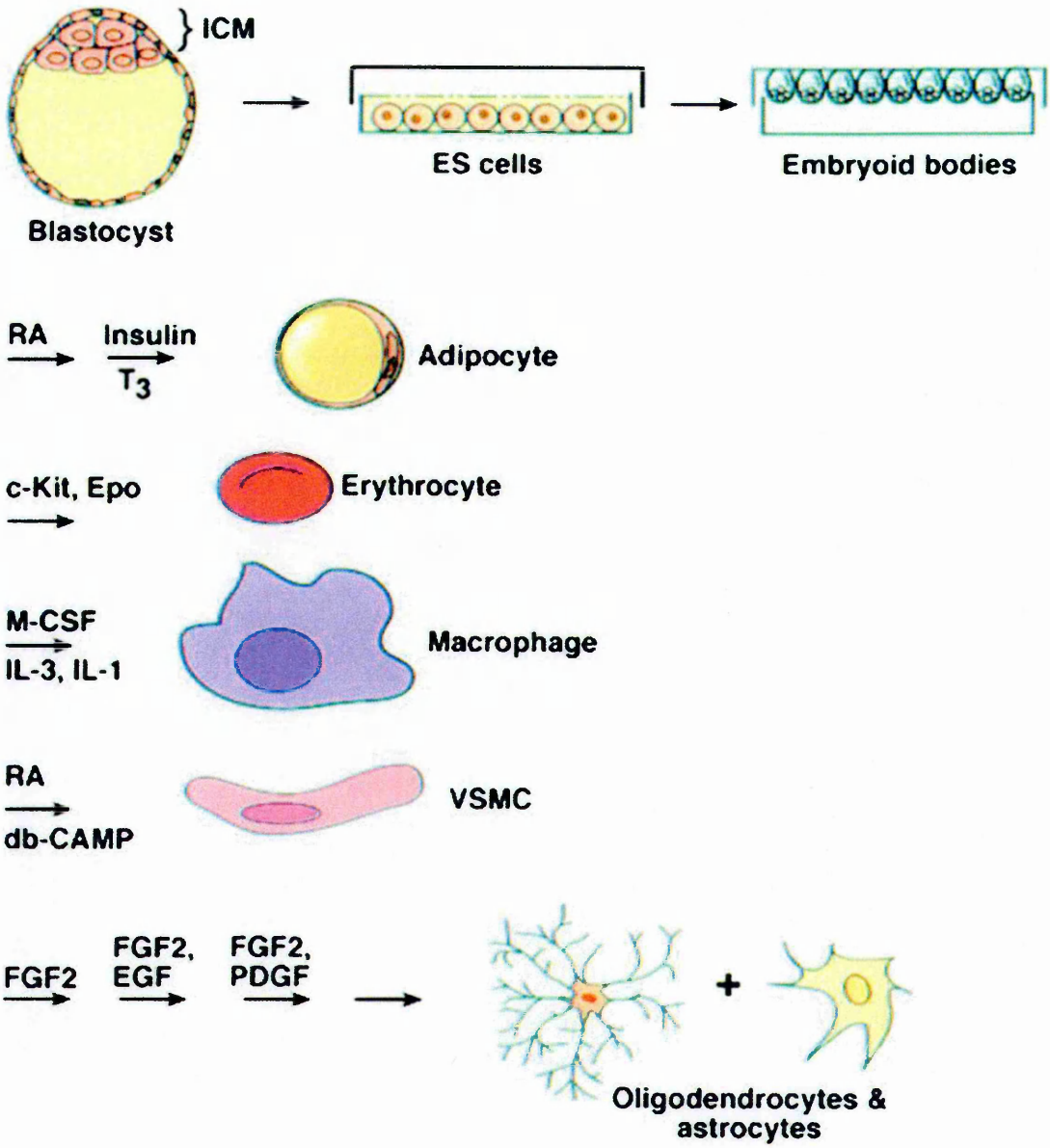

Figure 2. Differentiation Potential of Embryonic Stem (ES) Cells[7].

The pluripotency of the embryonic stem cells isolated from the inner cell mass of the blastocyst is not restricted to their potential to contribute to the formation of all tissues during development. Under the correct culture conditions, these cells can also differentiate in several cell types of the adult mouse, such as haematopoietic and neural cells.

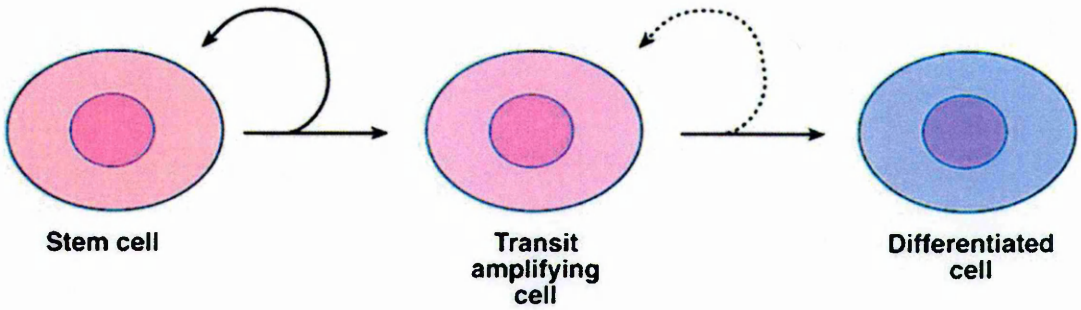

Figure 3. Mechanism of Action Adult Stem Cells[7].

The adult stem cells are found on the top of the lineage hierarchy and are committed to a specific cell lineage. Once they are activated, self-renew and/or proliferate to produce large numbers of transit amplifying cells. Finally, the propagation capacity of these cells is exhausted and they terminally differentiate to the desired cell type.

1.1.1 Haematopoietic Stem Cells.

The most studied adult stem cells are the haematopoietic stem cells (HSC). These cells were initially thought to remain quiescent until their activation and differentiation, but it is now known that they are slow-cycling cells. BrdU incorporation and cell cycle analysis experiments showed that 8% of them enter the cell cycle per day and 99% divide every 1–2 months (these frequencies are increased in older mice [8]). This continuous cell division necessitates the existence of a self-renewal mechanism for the maintenance of HSCs during lifetime [9]. The size of this reserved cell pool is also regulated by apoptosis, since the over-expression of the anti-apoptotic *Bcl*-2 in HSCs conferred protection against irradiation-induced cell death [10] and a repopulation advantage in comparison to wild type HSCs, as revealed by competitive transplantation experiments [11]. Notably, parabiosis experiments (during which the circulation of two mice is surgically joined) demonstrated that HSCs periodically leave their BM niche, travel in the circulation and return to the BM, in which they re-engraft [12]. This physiological migration is regulated by chemokine receptors (e.g. CXCR4) and adhesion molecules [13], like P- and E-selectin [14] and VCAM-1 (vascular cell adhesion molecule-1) [15].

HSCs are adult multipotent stem cells and give rise both to the red and all the white blood cells [16]. Remarkably, the transplantation of a single HSC into a mouse, whose own haematopoietic cells have been previously depleted by whole-body gamma irradiation, rescues the animal from anaemia-caused death. This is accomplished thanks to the ability of this single HSC to repopulate completely the haematopoietic system [17]. A similar practice is followed for the treatment of human patients affected by leukaemia. In this case, irradiation or cytotoxic drugs are administered to kill the tumour blood cells, which are subsequently replaced by the healthy donor cells [18].

The separation of several BM-derived subpopulations, using specific cell-surface proteins as markers and flow cytometry sorting, has allowed the fine characterization and distinction of the haematopoietic stem/progenitor cells according to their diverse *in-vivo* repopulating capacity and *in-vitro* differentiation properties. Today, we can recognize two populations that can be considered HSCs, the long-term (LT-HSC: Thy-1^{low}Lin$^-$Sca1^+Mac-1^-CD4$^-$c-kit$^+$) and the short-term (ST-HSC: Thy-1^{low}Lin$^-$Sca1^+Mac-1^{low}CD4$^-$c-kit$^+$) ones. They differ from each other in the length of the time period for which they can reconstitute the haematopoietic system of a mouse following irradiation and transplantation, reflecting their diverse self-renewal capacity. The LT-HSCs can provide the necessary haematopoietic cells and identical daughter LT-HSCs for the whole life of the mouse. On the contrary, the ST-HSCs are able to produce haematopoietic cells only for a limited period of time, and cannot generate any LT-HSCs but only daughter ST-HSCs and multipotent progenitors (MPP: Thy-1$^-$Lin$^-$ Sca1^+Mac-1^-CD4^{low}c-kit$^+$), which do not bear self-renewal ability at all [19]. Similar transplantation experiments allowed the identification of a common lymphoid progenitor (CLP, Lin$^-$ IL-7R$^+$Thy-1^-Sca-1^{low}c-Kit^{low} cells) and a common myeloid progenitor (CMP, Lin$^-$ IL-7R$^-$Thy-1^-Sca-1^-c-Kit$^+$ cells) that display lymphoid-restricted (T, B, and NK cells) [20] and myeloid/erythroid-restricted (megakaryocyte/erythrocyte or granulocyte/macrophage progenitors) [21] reconstitution and differentiation capacity, respectively (Figure 4).

The developmental origin of HSCs remains controversial. In mice, the first haematopoietic cells are detected in the yolk sac (YS) at embryonic day 7 (E7), as shown by the *in-vitro* detection of both erythroid and macrophage progenitors [22]. These cells are multipotent progenitors, since *in-vitro* cultures of clones derived from YS between E8.5 and E9.5 can give rise both to pre-B-cell and myeloid clones [23]. However, they cannot reconstitute *in vivo* the haematopoietic system of lethally irradiated adult

mice and therefore are not considered ancestors of HSCs. Between E7.5 and E10, another haematopoietic site exists in the paraaortic splanchnopleura/aorta-gonad-mesonephros region (PAS/AGM). AGM keeps its haematopoietic capacity for a few days and its relationship with YS is uncertain. *In-vitro* culture of cells isolated from PAS (E7.5-E9) resulted in the formation of B and T cell clones [24]. In addition, the transfer of AGM-derived cells (E10) into adult irradiated recipient mice accomplished the complete repopulation of the mice, demonstrating that this region contains cells with HSC activity [25]. Indeed, it is hypothesised that these cells initially colonise the liver (the most potent embryonic haematopoietic tissue) and finally the spleen and the BM (the definitive haematopoietic organ) after E15. Although, these findings suggest that the definitive HSCs appear already at E10 in AGM, it is still in doubt whether the adult BM-derived HSCs originate from these primitive cells with HSC characteristics or appear only later in development. This latter idea is supported by the distinct gene expression profile between the embryonic and adult haematopoietic cell populations [26].

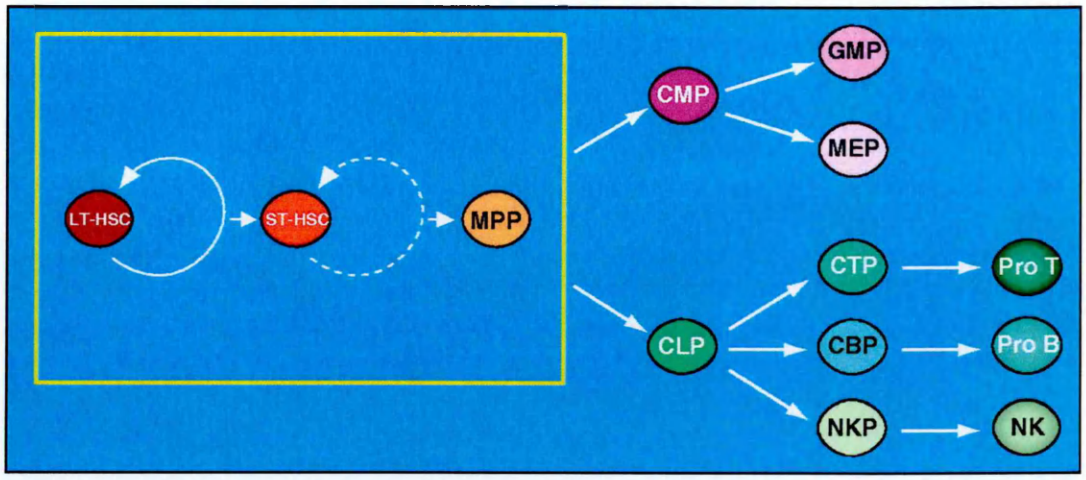

Figure 4. Model of HSC Differentiation [27].

The LT-HSCs can self-renew and/or generate ST-HSCs for the organism's lifetime. The ST-HSCs are also able to self-renew and produce multipotent, non-self-renewal progenitors (MPP), but for a limited time period. MPPs are the last type of cells in the hierarchy that can give rise both to the myeloid and the lymphoid lineage, and operate through intermediate progenitors called common myeloid progenitors (CMP) and common lymphoid progenitors (CLP), respectively. This cascade continues with the generation of more progenitors that progressively become more committed, e.g. myelomonocytic progenitors (GMP) and megakaryotic/erythroid progenitors (MEP) are the progeny of the CMPs, and finally ends with the differentiation of unipotent precursors into the desired cell types, e.g. B cells from the B progenitors (pro-B).

1.1.2 Muscle-resident myogenic stem/progenitor cells.

1.1.2.1 Satellite Cells.

At variance with HSCs, other adult stem cells are not so well studied. More recently, though, a great effort has been made to identify more adult stem cells and to investigate their mechanisms of action. The potential clinical applications of the muscle stem cells in diseases, like muscular dystrophy, has promoted the stem-cell research in muscle biology in the last decades. Remarkably, the skeletal muscle stem cells, also termed satellite cells, have been discovered since the 1960s. They are defined as the mononuclear cells located between the basal lamina and the plasma membrane of the adult-mouse myofiber and their role is the postnatal growth and the muscle repair (Figure 5) [28]. These cells have very high nucleus/cytoplasm and heterochromatin/euchromatin ratios and they contain a reduced number of organelles qualitatively and quantitatively, suggesting an inactive state [29]. Their distinct *in-vitro* differentiation characteristics [30] and gene expression patterns [31], compared with the embryonic and the foetal myoblasts ones, strongly suggest that they are indeed a separate cell population restricted to the late embryo and the adult mouse.

Unfortunately, the above spatiotemporal definition is not accurate enough and consequently satellite cells are not identical to each other but exist as a heterogeneous population with diverse myogenic potential; therefore not all the satellite cells are true muscle stem cells. This imprecision, in conjunction with the limited ability of these cells to regenerate the muscle after their transplantation [32], due to survival [33,34], immunological [33,35] and migration problems [36], had opened a debate whether or not the satellite cells should be considered stem cells. The formal proof of satellite cells being true stem cells was supplied only lately, by experiments that demonstrated their ability

to self-renew, repopulate irradiation-depleted muscles and regenerate the muscle greatly [37].

The confusion concerning the stem cell capacity of satellite cells has also been caused by the inadequate isolation methods used. Traditionally, the whole muscles were dissected and enzymatically disaggregated, leading to cell cultures containing not only satellite-cell-derived myoblasts, but also other myogenic cells and fibroblasts. Since the mid 1980s, a fine technique has been established, allowing the isolation of satellite cells from single myofibers, their expansion and their study *in vitro* [38]. This method does not avoid the problem of the internal satellite-cell heterogeneity, but at least prevents the interference from other populations. However, satellite cells exhibit best their myogenic potential and retain their stem-cell characteristics when they are isolated directly by flow cytometry sorting and immediately transplanted without any previous *in-vitro* manipulation [39].

The combination of the above experimental systems has recently elucidated several aspects of the satellite-cell biology. In the absence of stimuli satellite cells are quiescent; but during normal growth or muscle regeneration, induced by exercise, injury or degenerative diseases, become activated and enter the cell cycle, giving rise to two different cell types, the daughter myoblasts and the daughter satellite cells. This asymmetric cell division has been recently demonstrated by the asymmetrical co-segregation of the template DNA strand [40] and the membrane-associated cytoplasmic protein Numb [41], which had been previously related with cell-fate determination. Notably, the daughter satellite cells can return to quiescence and constitute a reservoir of stem cells for future use; while the resulting daughter myoblasts proliferate extensively and finally differentiate and fuse with the damaged myofiber or with each other, to repair or to create new muscle fibres, respectively [42].

This step-wise process is strictly regulated by the temporal expression of a category of transcriptional factors, called myogenic regulatory factors (Mrfs). They are proteins that contain a DNA-binding basic helix-loop-helix (bHLH) motif and they have been originally identified for their ability to induce the conversion of C3H10T1/2 fibroblasts into myoblasts [43]. More recently, another category of transcription factors, called paired box genes, has been associated with satellite cells. Specifically, *Pax7* and *Pax3* (paired box gene 3) that share high homology, are expressed by satellite cells. While *Pax7* is the most useful marker of quiescent satellite cells and is also transcribed by proliferating myoblasts, the synthesis of Pax3 is restricted only to satellite cells of some muscle-types [44]. Initially thought to be necessary for the myogenic specification of satellite cells [45], Pax7 was proven by later studies to be essential for their propagation and their maintenance via its anti-apoptotic activity [44,46,47].

Satellite cells in mitotic quiescence express the genes *c-met* [48], *CD34*, and *Pax7* [45]. The expression of *Myf5* (myogenic factor 5) within the satellite-cell population ($Pax7^+$ cells) is heterogeneous, since the 90% of the cells are $Myf5^+$ and the resting 10% $Myf5^-$ [49]; while the remaining Mrfs are not present. Interestingly, this heterogeneity is resulting from the asymmetric cell division of the $Pax7^+/Myf5^-$ cells and the *Myf5* expression in the daughter cells is determined by their basal or apical location. Remarkably, the intramuscular injection of $Pax7^+/Myf5^-$ and $Pax7^+/Myf5^+$ cells demonstrated that exclusively the $Myf5^-$ cells are able to reconstitute the satellite-cell pool *in vivo*, suggesting that the above cell populations should be considered true muscle stem cells and myogenic committed progenitors, respectively [49]. However, asymmetric cell division has previously been described also for the $Myf5^+$ cells [40,41], rendering unclear whether this event occurs prior or after the activation of Myf5. Nevertheless, these two possibilities are not mutually exclusive, and it is probable that the asymmetrical cell division takes place in both cell types, implying a multi-level

hierarchical model of muscle stem cell lineage progression, similar to the one accepted for LT- and ST-HSCs (see section 1.1.1).

In spite of the above discrepancy, it is recognised that the satellite cells, after their activation, start to transcribe the myogenic differentiation-1 gene (*MyoD*) and up-regulate Myf5, while they continue to express *Pax7* [48,50-53]. Their proliferation leads to the creation of a heterogeneous population, regarding their expression pattern for *Mrfs* and *Pax7* and hence their functional characteristics [54]. Two distinct subpopulations co-exist: one slow-cycling [55], of cells with round morphology [56] that retain *Pax7* expression but down-regulate MyoD, and a second one, of "thick" cells [56] that continue to express *MyoD*. The former one ($Pax7^+/MyoD^-$) does not differentiate but returns to the quiescent state and becomes the "reserve" cells, while the latter one ($Pax7^+/MyoD^+$) keeps on proliferating and is destined to terminally differentiate [50,57]. Consequently, these latter affirmations suggest an additional possible mechanism for the self-renewal of satellite cells.

Notably, *MyoD* over-expression converts the reserve cell population to differentiating cells, [57] and $MyoD^{-/-}$ primary myoblasts display reduced differentiation potential and their transition from proliferation to differentiation is delayed *in vitro* [58]. *In vivo*, the muscle fibre formation in $MyoD^{-/-}$ mice is delayed during muscle regeneration [59]. Therefore, despite that MyoD appears early, it is probably more important for the differentiation of satellite cells. After several rounds of proliferation and once a sufficient number of myoblasts is reached, the $MyoD^+$ cells stop to proliferate and start to differentiate. This stage coincides with the down-regulation of *Pax7* and the expression of two more *Mrfs*, *myogenin* and *Mrf4* (myogenic regulatory factor 4) [52,57,60,61]. Following the expression of these late Mrfs, early muscle differentiation genes, such as *myosin heavy chain* (*MHC*), *myosin light chain* (*MLC*) and *muscle creatine kinase* (*MCK*), are transcribed. Finally, the late muscle

differentiation proteins (e.g. dystrophin and δ-sarcoglycan) that are necessary for the contractile function of the myofibers [42] are produced (Figure 6).

The developmental origin of satellite cells, like the most aspects of their biology, has been uncertain until very recently. Initially, transplantation studies of donor quail somites into host chicken embryos had suggested that satellite cells have the same somitic mesodermal origin with all the embryonic, foetal and adult skeletal muscle [62]. Nevertheless, later experiments with explants from the dorsal aorta or the somites (E9.5) resulted in the generation of myoblast clones only in the dorsal-aorta-derived cultures, while the somite-derived ones had a fibroblast-like morphology. These dorsal-aorta-derived round-shaped clones were able to differentiate to myosin positive cells and to participate in muscle regeneration after their intramuscular injection. In addition, satellite cells appear also in the *Pax3*$^{-/-}$ and *c-met*$^{-/-}$ mice that lack the somitically-derived migrating myogenic progenitors. Although, these data indicated that the developmental source of satellite cells is not somitic, but endothelial and differs from the other myogenic cell lineages [63]; it is now believed that the extent of the non-somitic contribution is limited and does not represent a major source for satellite cells. Instead, recent quail-chick grafting experiments, showing that Pax7^{+} satellite cells originate from the dorsal compartment of the somite [64,65], and developmental studies, employing *Pax3*$^{GFP/+}$ and Pax7$^{LacZ/+}$ and double transgenic mice, re-proposed a common somitic origin for all the myogenic lineages, including the satellite cells [66]. The above two hypotheses are not necessarily mutually exclusive and the heterogeneity in the satellite-cell compartment could also be explained by the presence of two distinct cell populations with separate origins (Figure 7).

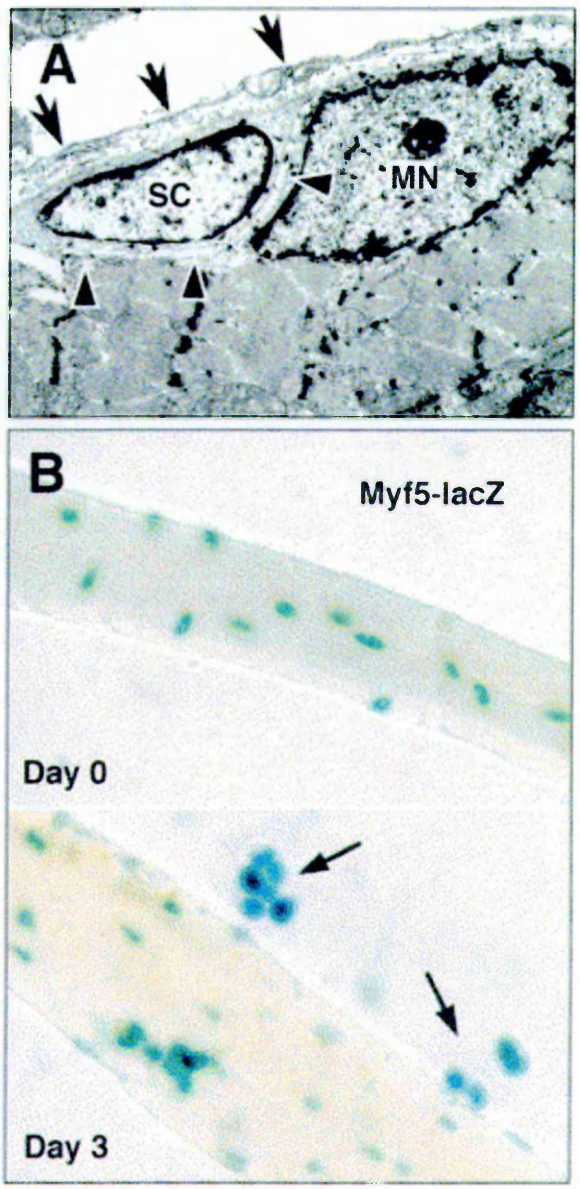

Figure 5. Anatomical position of satellite cells [67].

(a) Electron microscopy reveals the anatomical position of satellite cells *in vivo*. A satellite cell (SC) is located in the sublaminal space, between the membrane of the fibre and the basal lamina as indicated by the arrowheads and arrows, respectively. Adjacent to it, a myonucleus (MN) within and at the periphery of the myofiber can be observed. **(b)** X-gal staining of a single muscle fibre, isolated from a *Myf5*$^{nlacZ/+}$ knock-in mouse, reveals several Myf5^{+} (satellite) cells attached on it (day 0). Three days after their isolation (day 3), activated Myf5^{+} satellite-cell-derived cells are found detached from the fibre.

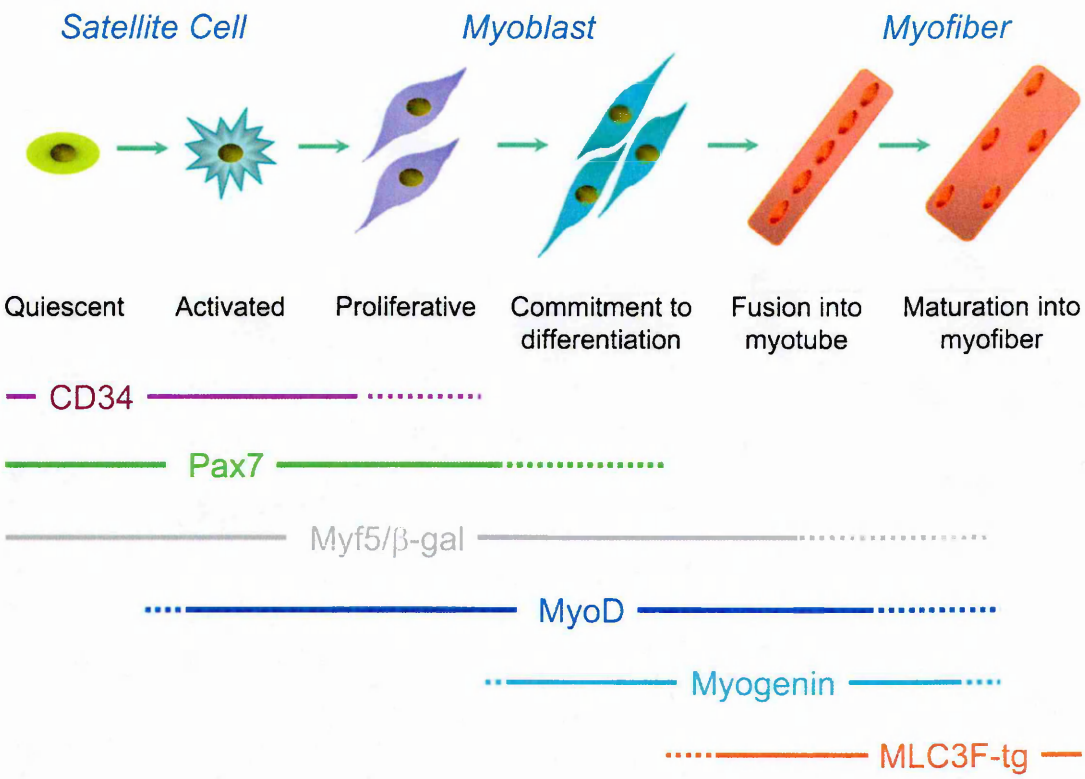

Figure 6. Temporal expression of myogenic marker genes by satellite cells [68].

CD34 and *Pax7* are expressed in all the quiescent satellite cells; while the expression of *Myf5* is heterogeneous, as revealed by the usage of a knock-in ($Myf5^{nlacZ/+}$) mouse that produces δ-galactosidase under the control of the Myf5 promoter. Activation coincides with Myod synthesis, which becomes permanent in contrast to Pax7 that is down-regulated during differentiation and Myf5 that is detected until the early stages of fusion. The expression of myogenin starts at the beginning of the differentiation and afterwards never ceases. MLC, as shown by the activation of the MLC promoter in the MLC3F-*nlacZ*-E transgenic mice, and other structural proteins are detectable at the late differentiation.

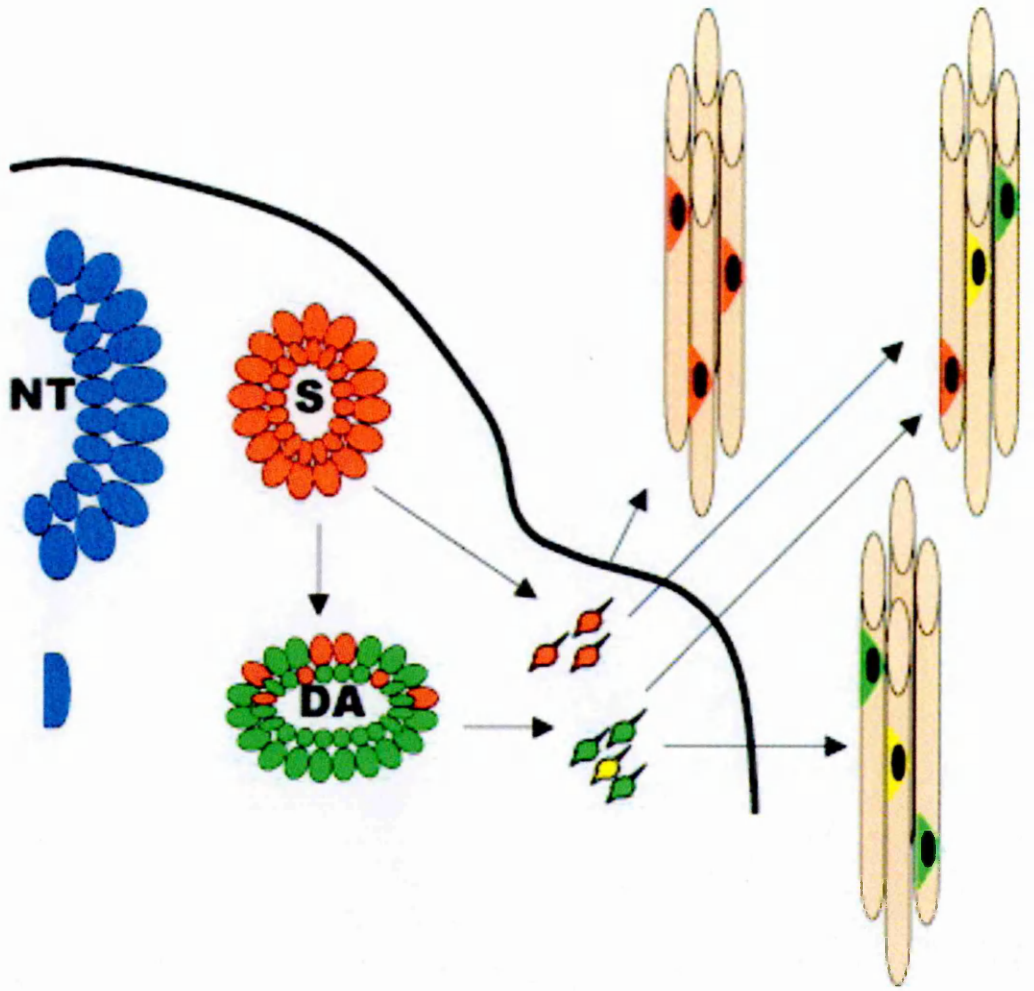

Figure 7. Developmental origin of satellite cells [69].

The two possible origins of satellite cells are schematically represented. According to the first one, the somite (S) gives rise to all the myogenic progenitors (red cells), including the migrating cells that will constitute the satellite-cell compartment in the adult mouse. Other reports suggested that satellite cells derive from muscle precursors (green cells) located in the dorsal aorta (DA). However, it cannot be excluded that the DA-derived precursors themselves originate from the somite (red cells) and subsequently generate the quiescent satellite cells (yellow cells). Overall, the data convincingly demonstrate that the myogenic progenitors, with satellite-cell behaviour, can be isolated both from the somite and the dorsal aorta. Indeed, it is probable that the satellite cell pool has a mixed source that could explain its heterogeneity.

1.1.2.2 Non-satellite muscle-resident myogenic progenitors.

In addition to the satellite cells several other muscle-resident myogenic progenitors have been identified. One of them is the side population (SP) cells that are defined for their ability to actively exclude the hoechst 33342 fluorescent dye. These cells cannot differentiate into myocytes in culture alone, but proliferate in response to muscle injury [70], become specified to myogenesis in co-culture with myoblasts [65,70,71] or C2C12 cells [72] and differentiate when exposed to Matrigel matrix [73]. Intravenous injection, in dystrophic mice, of muscle-derived SP (ms-SP) cells that are $Sca1^+/CD45^-/c\text{-}kit^-$, although this phenotype was contradicted by later studies (see below), demonstrated that they can incorporate into the muscle fibre and express *dystrophin* [74]. This ability was also confirmed by other studies that employed intramuscular [70,71,73] or intravenous [72] injection of ms-SP cells in regenerating or dystrophic mouse muscles, respectively.

The muscle-resident $CD45^+/Sca1^+$ and $CD45^-/Sca1^+$ cells are also increased and proliferate during muscle regeneration [75], and display *in-vivo* myogenic potential with low frequency and high frequency, respectively [76]. The latter cell population can differentiate in culture alone [76,77], while $CD45^+/Sca1^+$ cells cannot and need to be induced by different factors like muscle regeneration, co-culture with myoblasts, ectopic expression of *Pax7* or activation of the Wnt signalling pathway by LiCl treatment [75,76,78], calling to mind the self-insufficient myogenesis of ms-SP cells. This similarity, between the muscle-resident $CD45^+/Sca1^+$ and the ms-SP cells, indicates a link between the two cell populations and indeed, this case cannot be excluded, since the CD45 and Sca1 phenotype of the muscle-resident SP cells is controversial. Although it had been initially reported that they express *Sca1* but not *CD45* and *c-kit* [74]; nowadays it is generally accepted that they comprise a heterogeneous cell population, including mainly $CD45^-/Sca1^+/c\text{-}kit^-$ cells but also $Sca1^-$, $c\text{-}kit^+$ and $CD45^+$ cells and all

the possible combinations of cell subpopulations [65,71,73,74,79-81], making any attempt to compare studies investigating muscle-resident SP cells and $CD45^+/Sca1^+$ cells chaotic. Nevertheless, both of them lack an intrinsic and signal-independent myogenic potential, indicating the importance of the microenvironment for their myogenic differentiation.

Muscle progenitors have also been isolated by pre-plating muscle preparations and subsequent flow cytometry sorting for the CD34 and the Sca1 markers. These cells, termed muscle-derived stem cells (MDSCs), should be distinct from satellite cells, since the latter ones are traditionally thought to be negative for Sca1 [45,71,82], although rare satellite cells expressing *Sca1* have been detected [83]. However, MDSCs can be found within the basal lamina and they spontaneously differentiate in myocytes and myotubes *in vitro* [84]. Their intrarterial [85] or intramuscular [84,86] injection, in naturally occurring mutant mice lacking the dystrophin gene (*mdx* mice), demonstrated that they can contribute to the myofiber formation and express *dystrophin.*

A population of myogenic cells, which is certainly worthy mentioning for its potential clinical application, are the mesoangioblasts. These cells were initially isolated from the aorta of quail and mouse embryos, and their transplantation into chick embryos demonstrated their mesodermal multipotency, giving rise to chondrocytes and haematopoietic cells and contributing to the generation of vessels, bone and last but not least to skeletal and cardiac muscles [87]. Nowadays, mesoangioblasts can be also isolated from the vessels of the adult mouse [88], dog [89] and human [90,91] muscles, and therefore are also called vessel-associated stem cells. RT-PCR analysis revealed the expression of *CD34*, *Flk1* and *c-kit* but not *Myf5* and *MyoD* from freshly isolated mouse cells. Importantly, these stem cells can be expanded *in vitro* and differentiate into adipocytes and osteoblasts in culture alone; while co-culture with myoblasts, cardiomyocytes or BM cells is needed for their differentiation into myocytes, cardiocytes or haematopoietic cells, respectively [87]. Their intrarterial injection in δ-sarcoglycan null

mutant mice resulted in δ-sarcoglycan^{+} muscle fibres with improved morphology and functional properties, suggesting that they can regenerate the muscle at therapeutic levels [88]. This ability was increased, enhancing their migration into the muscle tissue, using a treatment with stromal derived factor-1 or tumour necrosis factor δ (TNFδ) or ectopic expression of δ4-integrin [90]. Recently, Sampaolesi and colleagues reported that the transplantation of mesoangioblasts in dystrophic dogs improved the function and the morphology of their muscles [89]. This last report in conjunction with the availability of these cells in humans [90,91] renders them a promising approach for the treatment of human dystrophies.

The relationship between the muscle-derived non-satellite myogenic progenitors and satellite cells is still unclear. Satellite cells, isolated from single myofibers, can generate myogenic but not haematopoietic clones, *in vitro* [71]. Conversely, the muscle-derived SP and CD45^{+}/Sca1^{+} cells cannot give rise myoblast clones but have *in-vitro* and *in-vivo* haematopoietic potential (see above and section 1.2.2). Based on these functional characteristics, it is clear that satellite cells and the muscle-derived SP and CD45^{+}/Sca1^{+} cells are distinct cell populations. However, studies claimed that intravenously [72,74] or intramuscularly [71] transplanted ms-SP cells can give rise to satellite cells, detected *in vivo* and in single muscle-fibre cultures. In addition, Seale and colleagues observed that in Pax7$^{-/-}$ mice the muscle cells exhibit an increased haematopoietic potential [75] and the muscle-derived CD45^{+}/Sca1^{+} (ms-CD45^{+}/Sca1^{+}) cells, although raised in proportion, are impaired for myogenesis, and therefore they deducted that the muscle-resident CD45^{+}/Sca1^{+} cells are a continuous source of satellite cells via a Pax7-dependent path [78]. Although these evidences had supported a hypothesis, according to which satellite cells are not the true muscle stem cells but they are another intermediate cell type (like myoblasts) between a multipotent stem cell and the terminally differentiated fibre; later studies confirmed that satellite cells are the true

muscle stem cells, based on their massive capacity to regenerate the muscle and their ontogeny (see section 1.1.2.1). Most probably, several myogenic progenitors exist in the muscle, both inside and outside of the satellite-cell compartment; but at the moment there are sufficient evidence only for the stem-cell ability of satellite cells (see Table 2).

Cell Population / Characteristic	Satellite cells	SP cells	CD45$^+$ /Sca1$^+$	CD45$^-$ /Sca1$^+$	CD34$^+$ /Sca1$^+$	Meso-angioblasts
Myogenic clones forming ability	+	-	-	+	+	-
Differentiation in culture alone	+	-	-	+	+	-
Differentiation in co-culture with myogenic cells	N/D	+	+	N/D	N/D	+
***In vivo* myogenic potential**	+	+	+	+	+	+
Haematopoietic clones forming ability	-	+	+	-	N/D	-
***In vivo* haematopoietic potential**	-	+	+	-	N/D	+

Table 2. Myogenic and haematopoietic potential of muscle-resident stem/progenitor cells.

1.2 Stem cell plasticity.

Cell plasticity and pluripotency are traditionally considered properties of the embryonic stem cells. On the contrary, the adult stem cells are thought to be restricted in their differentiation potential to the characteristic progeny of the tissue in which reside (see section 1.1). However, in the last years several reports suggested that adult stem-cell plasticity also exists (Figure 8). For example, neural cells contributed to bone marrow (BM) [92] and to muscle [93], muscle to BM [94] and pancreatic cells to liver [95]. BM cells appear to show the highest level of plasticity, participating in the formation of liver [96], lung [97], skin [97], kidney [98], pancreas [99], myocardium [100], central nervous system (CNS) [101] and skeletal muscle [102]. In all cases, the differentiation of stem cells into a non-canonical progeny is an exceedingly rare phenomenon, almost invariably associated with severe damage in the target tissue and often with a specific selective pressure. Despite the low frequency of the phenomenon, these observations suggested that the traditional view of tissue-specific adult stem cells may need to be revised and that the adult stem cells may not act only locally but they might also be recruited and reprogrammed.

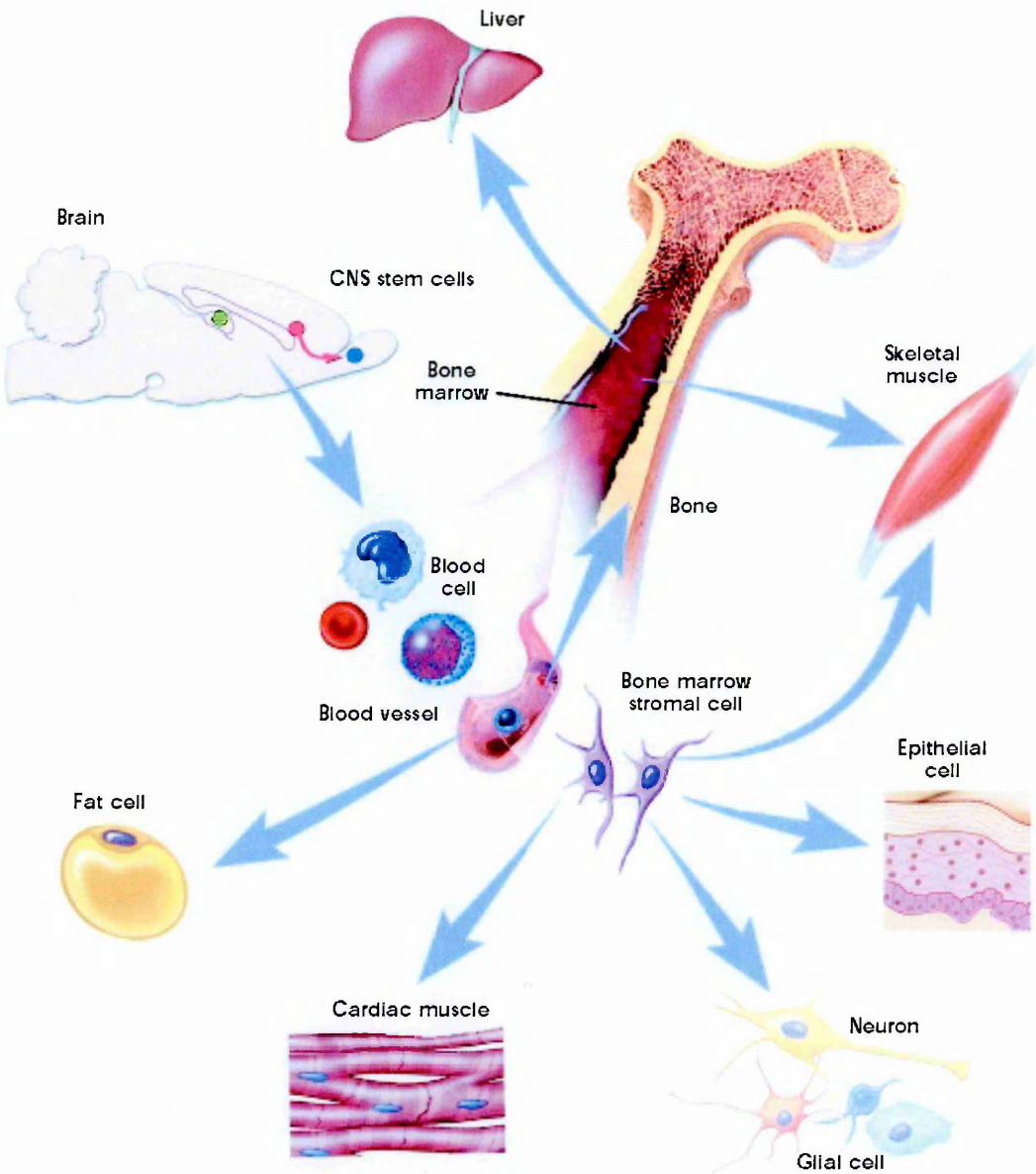

Figure 8. Stem cell plasticity [103].

Schematic representation of the ability of adult stem cells to generate cell types different from the ones of the tissue in which reside. The traditional view of stem cell biology contends that the adult stem cells are committed to the lineage in which reside. This dogma alleges that their nuclei have undergone irreversible chromatin changes that forbid the activation of other non-tissue-related transcriptional programs. In the last years, several experiments have proven that the nuclear reprogramming of the adult stem cells, thanks to chromatin plasticity, is possible; although its extent remains to be defined.

1.2.1 Bone Marrow-derived myogenesis.

The role of BM-derived cells in adult-muscle regeneration is probably the most extensively studied case of adult stem-cell potential plasticity. The phenomenon was initially described in experiments, where non-fractionated BM cells were injected into damaged mouse muscles or muscle regeneration was induced in BM-transplanted mice. In both cases, BM-derived cells participated in the formation of new muscle fibres [102]. Notably, the infrequency of the event did not allow the correction of murine muscular dystrophy by BM transplantation, casting doubt over the very existence of BM-derived myogenesis [104]. However, numerous reports describing several methods of cell delivery (BM transplantation, intramuscular injection or even parabiosis) and induction of muscle regeneration (cardiotoxin, myotoxin injury or long-term exercise) confirmed its occurrence [105-117]. This phenomenon is not well understood and there is still the need to investigate the nature of the BM subpopulation responsible for the event, the migration of these cells to the skeletal muscle, the existence of a myogenic program that regulates the whole process and the ability of the BM-derived nuclei located in the myofiber to be reprogrammed.

1.2.1.1 Characterisation of the Bone Marrow subpopulation responsible for the phenomenon.

The identification of the BM subpopulation, which can participate in muscle regeneration, is probably the best-studied aspect of BM-derived myogenesis. BM consists of two major and separate cell compartments, the stromal and the haematopoietic cells that can both contribute to the muscle fibre formation and are easily distinguished by the panhaematopoietic marker CD45. The stromal-derived mecenchymal stem cells (MSC) do not express the haematopoietic markers *CD45* and

CD34 and exhibit increased adhesive properties, in contrast to the haematopoietic cells, allowing their isolation from mouse and human BM.

The MSCs are fibroblast-like cells, multipotent [118] and can differentiate in muscle *in vitro*, as shown by their co-culture with myoblasts [119] or C2C12 cells [120], or culture in conditioned medium prepared from injured muscle [121]. In addition, their injection into regenerating [120] or dystrophic [119] mouse muscles resulted in their incorporation into the muscle fibres and expression of *dystrophin*, demonstrating their ability to participate in muscle regeneration and to express muscle-late-differentiation genes *in vivo*. The activation of the Notch signalling in the BM-derived MSCs makes them potent myogenic progenitors. Most of the MSCs, ectopically expressing the intracellular domain of Notch, differentiated in $Pax7^+$/$MyoD^+$/$myogenin^+$ myocytes and myotubes *in vitro*. Following intravenous or intramuscular injection, they regenerated the muscle and gave rise to $Pax7^+$ cells located in the sublaminal space, strongly suggesting that they become satellite cells [122].

A pluripotent subpopulation of MSCs, termed multipotent adult progenitor cells (MAPCs) has been also isolated [123], using a 3-week culture period and exclusion of all the remaining haematopoietic cells ($CD45^+$ and $Ter119^+$ cells). Like ES cells (see section 1.1), MAPCs have the remarkable ability to contribute to most somatic tissues, including the muscle, following their transplantation into early blastocysts [124].

The haematopoietic cells are also able to contribute in muscle regeneration, but in contrast to MSCs they have the additional ability to circulate. Transplantation of single HSCs (BM-derived $CD45^+$/$Sca1^+$) or BM-derived $CD45^-$ cells has revealed that exclusively the haematopoietic subpopulation ($CD45^+$) contains circulating BM cells with potential myogenic activity and that one single HSC is enough to sustain the BM contribution to skeletal muscle [106]. Similar results were obtained by the transplantation of a single BM $c\text{-}kit^+$/Lin^-/$Sca1^+$ (HSC) cell [125] or BM SP cells [74], which are purified for

their ability to exclude the Hoechst 33342 dye, are enriched for HSCs ($CD45^+/Sca1^+/c\text{-}kit^+/lin^-$ cells) and have been also detected in the muscle tissue (see sections 1.1.2 and 1.2.2). These findings, supported by numerous later studies [109,111,126], demonstrated that BM-derived myogenesis, following BM transplantation, results from the incorporation of haematopoietic cells into the muscle fibre and not from the differentiation of mesenchymal stem cells. However, the above experiments did not clarify if HSCs themselves or one of their daughter lineages fuse to the muscle fibre.

More recently, the intramuscular injection of several haematopoietic cell subpopulations, based on the expression of the cell surface markers CD45, c-kit, Sca1 and other haematopoietic lineage markers, has allowed a detailed characterization of the haematopoietic cells that can participate in muscle regeneration. Importantly, none of the mature lymphoid or myeloid populations were able to contribute to myofibers formation, but fractions containing HSCs and myeloid progenitors readily incorporated into the muscle fibres, suggesting that BM-derived myogenesis is due to the plasticity of haematopoietic stem/progenitor cells [107]. This notion was also strengthened by concurrent studies, where BM-derived $SP/CD45^+/Mac1^{low}$ cells (early myeloid precursors) but not $SP/CD45^+/Mac1^{high}$ (mature myeloid cells, macrophages) cells, isolated from the injured muscles of BM transplanted mice, were able to contribute to myotubes formation in co-culture with myoblasts [110] (see Table 3).

Cell Population / Characteristic	MSCs	MAPS	HSCs	CMPs	Mature haematopoietic cells
Myogenic clones forming ability	-	-	-	-	-
Differeniation in culture alone	-	-	-	-	-
Differeniation in co-culture with myogenic cells	+	+	+	+	-
***In vivo* myogenic potential**	+	+	+	+	-
Ability to circulate	-	-	+	+	-

Table 3. Myogenic potential of the BM-derived cells.

1.2.1.2 Migration of Bone Marrow cells to the muscle.

The homing signals that recruit the BM cells, which incorporate into the myofiber, seem to be the same signals to which the cells of the immune system (e.g. white blood cells) respond during inflammation, since the percentage of the BM-derived cells with myogenic potential ($CD45^+/Sca1^+$ [127] and $SP/CD45^+$ [110]) and the number of myofibers containing BM-derived nuclei [105,110,117,125] are readily increased after muscle damage. These observations are not surprising, since both cell types have a haematopoietic origin and reach the damage point shortly after injury. Additionally, some of these signals are also important for the regulation of satellite cells, showing that the processes of inflammation and regeneration are closely linked and currently it is impossible to dissect one from the other [42].

Insulin-like growth factor-1 (Igf1), which promotes the satellite-cell activation and proliferation and is upregulated during muscle regeneration [113], is of particular interest. Its local production in muscles of transgenic mice, previously transplanted with BM SP cells and in which *Igf1* is under the control of the muscle-specific promoter MLC, increases the muscle recruitment of the BM/donor-derived $CD45^+/c\text{-}kit^+$ and $Sca1^+/c\text{-}kit^+$ cells [128] that are known for their myogenic potential. In more recent studies, Sacco and colleagues [113] used DNA electrotransfer, myoblast-mediated delivery or direct injection of Igf1, in order to achieve increased levels of Igf1 in muscles of previously BM-transplanted mice. This approach led to a considerable increase in the contribution of BM cells in the muscle-fibre formation, demonstrating the importance of Igf1 in BM-derived myogenesis.

Contemporaneously, *in-vitro* migration assays showed that the migration of BM-derived haematopoietic progenitors towards C2C12 or primary myoblast-derived

myotubes is reduced by specific-treatment with an antibody blocking the receptor (Met) of the hepatocyte growth factor (Hgf), indicating that BM-derived cells migrate to the muscle in response to Hgf [129]. Interestingly, this growth factor regulates muscle-cell migration from the somites to the limbs and to the diaphragm during mouse development [130] and it is tempting to speculate that the BM-derived cells with myogenic potential follow the same path with the embryonic myogenic progenitors. Paradoxically, *Hgf* is not expressed in the adult, both in healthy and injured muscles, as revealed by a gene expression profiling monitoring the process of muscle regeneration [131].

1.2.1.3 Fusion versus Transdifferentiation.

Whether a specific myogenic program regulates BM-derived myogenesis, or it is simply the result of cell fusion is a controversial subject. The multinuclear nature of myofibers complicates any study that aims to investigate this aspect of the phenomenon, since fusion is the physiological manner to create muscle fibres and must take place for BM cells participating in muscle regeneration. Hence, the correct question is not if fusion occurs, but if a myogenic molecular program is activated within the BM-derived progenitors prior to their fusion to the muscle fibre. Intravenously transplanted BM cells were found between the plasma membrane of the muscle fibre and the basal lamina, where satellite cells (see section 1.1.2.1) are normally located, expressed satellite-specific genes, such as *Myf5*, *c-met*, *Pax7*, *M-cadherin*, or *CD34* [105,108,126,127] and proliferated in the damaged muscles [105]. BM-derived cells isolated from the muscle of the recipient mice formed clones that expressed the satellite marker desmin, and were able to differentiate in multinucleated myotubes *in vitro* or fused with muscle fibres after their injection into tibialis anterior (TA) mouse muscles [105]. Interestingly, the cultures of single muscle fibres from long-term BM-transplanted mice revealed the presence of BM-derived cells able to contribute to myotubes formation [126]. All the above reports indicated that haematopoietic cells not only incorporate to the muscle fibres but more importantly they can give rise to satellite cells.

Sherwood and colleagues [127] confirmed the ability of BM cells to migrate and engraft in the sublaminal space, and express *M-cadherin*, and *CD34*; but in the same study they failed to reproduce the previously described intrinsic *in-vitro* colony-forming ability or myogenic differentiation (as assayed by *MHC* expression), displayed by these cells. In addition, the presence of BM cells within the basal lamina, expressing *M-cadherin* [114], and the occurrence of BM-derived satellite cells in single myofiber

cultures [115] were also challenged, and it was suggested that haematopoietic cells participate in muscle regeneration by direct fusion without any previous specification to a muscle progenitor [106]. These contradicting reports have fed a debate between transdifferentiation and fusion that remains open up today (Figure 9).

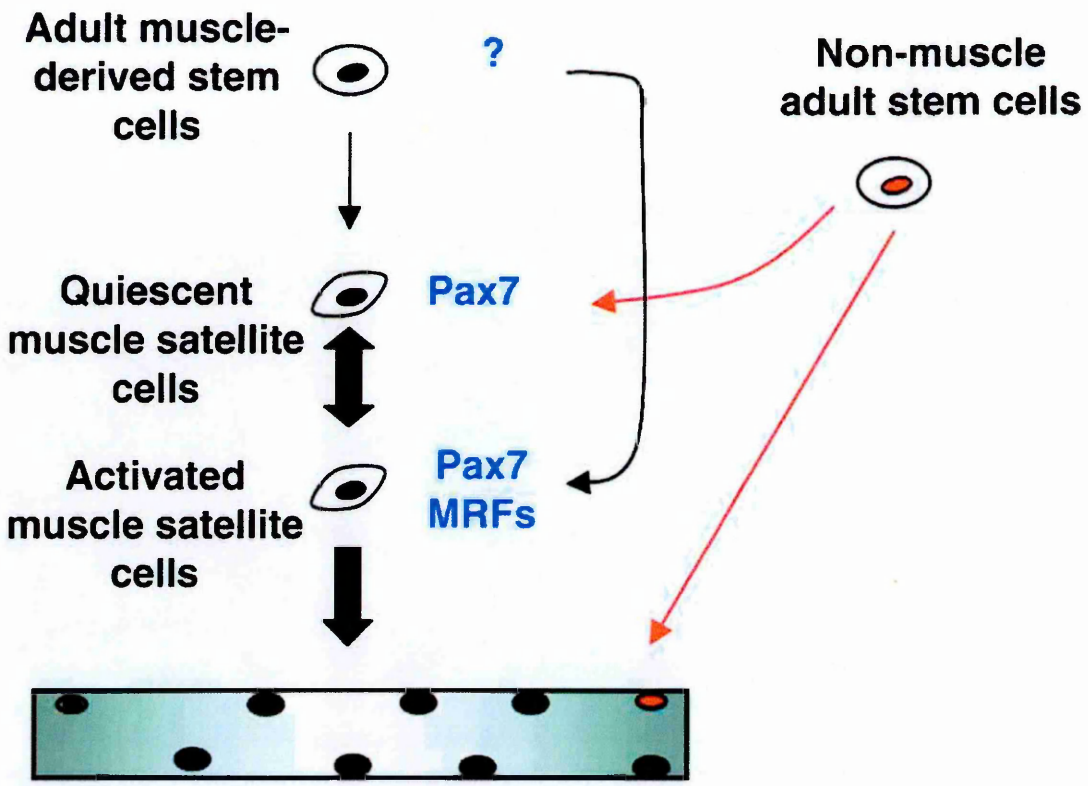

Figure 9. Mechanisms of BM-derived myogenesis [42].

The molecular mechanism that regulates the participation of BM cells to muscle regeneration is the most interesting and controversial aspect of BM-derived myogenesis. Studies have claimed that certain BM-derived cells (cell with red nucleus) can become identical to the satellite cells (mononuclear cell with black nuclei), activating the same myogenic program used by the latter ones (e.g. Pax7 and Mrfs), and that the nuclei of these cells are completely reprogrammed (black nuclei). A second hypothesis predicts that these BM cells (cell with red nucleus) do not express any of the muscle-specific genes but simply fuse to the myofiber and therefore their nuclei are not reprogrammed (red nucleus into the fibre).

1.2.1.4 Nuclear reprogramming.

Although the property of haematopoietic cells to incorporate into muscle fibres is now generally accepted, the ability of these cells to be reprogrammed prior or after the fusion event is unclear. The first study, in which BM cells isolated from a transgenic mouse that expresses LacZ under the muscle-specific MLC3F promoter were delivered by intravenous or intramuscular injection, reported that BM-derived nuclei located within the regenerating myofibers were δ-gal$^+$, indicating that these nuclei started to express muscle-specific genes [102]. In addition, following reports claimed the synthesis of more muscle-specific proteins, like Myf5, myogenin [100] and dystrophin [74], by these incorporating cells, suggesting that a complete nuclear reprogramming is part of the process. However, further transplantation experiments of normal BM SP cells in *δ-sarcoglycan* null mutant mice demonstrated that almost all the incorporated donor nuclei fail to express the *δ-sarcoglycan* (*Sgcd*) gene [109]. In a similar experimental set-up, using the *dystrophin* mutant mice, only 30% of the muscle fibres, containing donor-derived nuclei, were dystrophin$^+$ [114]. Interestingly, a study using in parallel dystrophic- and laminin-δ2-deficient mice as recipients revealed that BM transplantation restores the laminin-δ2 but not the dystrophin expression [116], indicating that the myogenic reprogramming can be gene-dependent. These data clearly put in doubt the ability of BM cells to be totally reprogrammed and therefore this aspect of BM-derived myogenesis remains unclear.

1.2.2 Muscle-derived haematopoiesis.

The relationship between the muscle tissue and the haematopoietic system has been strengthened by the observation that the former one contains cells that have HSC characteristics. Ms-SP cells not only participate in muscle regeneration (see section 1.1.2.2) but are also capable of reconstituting the haematopoietic system upon their transplantation into lethally irradiated mice [74]. A more detailed and long-term study showed that the muscle-derived haematopoietic cells are indeed true HSCs, since they could give rise to all the haematopoietic lineages (i.e. B-, T-lymphocytes and myeloid cells) both in primary and secondary recipient mice. Unexpectedly, the authors of the same report, using competitive BM transplantations, claimed that the muscle tissue contains more HSCs than the whole BM (WBM). However, this experimental protocol included a culture period of the muscle cells prior to their transplantation, and this could enrich for HSCs, leading to an over-estimation of their frequency [94].In fact, fresh muscle preparations gave rise to haematopoietic colonies *in vitro* at a much lower frequency than the WBM [76,132].

The idea of muscle-derived non-haematopoietic cells with HSC characteristics was initially considered another proof of stem cell plasticity and this was temporarily supported by a report, in which $CD45^-/Sca1^+/c\text{-}kit^-$ cells isolated from the muscle of neonatal mice contained long-term *in-vivo* haematopoietic potential [133]. However, contemporaneous and later studies, fractionating muscle cells for the expression of the HSCs surface markers *CD45* and *Sca1*, showed that exclusively the $CD45^+$ cells can generate haematopoietic colonies *in vitro* [76,77,129]. These $CD45^+$ cells were both $Sca1^+$ and $Sca1^-$, but only $CD45^+/Sca1^+$ cells displayed multilineage haematopoietic engraftment *in vivo* [76]. The *CD45* expression and the haematopoietic potential of muscle-derived cells were also correlated by other studies, where muscle-derived

SP/CD45$^+$/Sca1$^+$ and SP/CD45$^+$/Sca1$^-$ cells (although at a lower frequency) produced haematopoietic colonies [71], while muscle-derived SP/CD45$^-$ cells did not [71,80,81]. McKinney-Freeman and colleagues characterized further this population, showing that muscle SP cells expressing *CD45* and *c-kit* are responsible for all the muscle-derived HSC activity [80].

Remarkably, parabiosis experiments demonstrated that the above muscle-resident SP/CD45$^+$/c-kit^{dim}, exhibiting multilineage and long-term haematopoietic repopulation capacity *in vivo*, could originate from the circulation [134]. Interestingly, numerous BM transplantation experiments clearly demonstrated that the muscle-residing CD45$^+$ cells are depleted by irradiation [129] and are subsequently replenished by BM cells [70,77,80,110,129,132]. Analysis of the chimaeric mouse muscles showed that the cells bearing *in-vitro* [129,132] and *in-vivo* haematopoietic potential [132] originated from BM. All these results strongly suggested that the haematopoietic potential exhibited by the muscle tissue is due to the BM-derived cells able to migrate and engraft to the muscle and it should not be attributed to the plasticity of the muscle-derived stem cells.

2 Aims and Approach

The BM-derived $CD45^+/Sca1^+$ cells are haematopoietic stem/progenitor cells that have the ability to circulate, migrate and engraft to the muscle tissue, and therefore they are of particular interest. Notably, these cells retain their haematopoietic potential, as revealed both by *in vitro* and *in vivo* (see section 1.2.2) assays; but they also acquire myogenic potential, as shown by their ability to participate in muscle regeneration (see section 1.2.1.1). Whether, this latter remarkable ability is the result of the reprogramming of BM-$CD45^+/Sca1^+$ cells and the activation of a myogenic molecular program within these cells, remains controversial (see section 0). This study aims to clarify this aspect of the process, investigating the role of the muscle microenviroment and key myogenic transcription factors.

We functionally characterized factors affecting BM-derived myogenesis, employing reporter assays for muscle-specific genes, a comparative DNA microarray analysis of $CD45^+/Sca1^+$ cells, deletion studies of myogenic transcription factors and ectopic expression of *Pax7*. Our study was focused on the myogenic transcription factors Pax7, MyoD and Myf5. In order to evaluate their role in the myogenic potential of HSC-derived progenitors, we set up a BM-myoblast co-culture system, which allows scoring the myoblast-clone-forming ability and the ability to undergo myogenic specification and differentiation *in vitro*, and we also used BM transplantations to assess BM-derived myogenesis *in vivo*. To this end we used labelled haematopoietic cells, in which reporter genes are under the control of the MLC, the Myf5 or a chimeric LTR/MCK promoter, or Pax7 and MyoD are missing. BM transplantation experiments in $Pax7^{-/-}$ recipient mice were also used to avoid the competition by the recipient's muscle stem cells and hence to fully explore the myogenic potential of the haematopoietic cells. In addition, a comparative gene expression profiling of the BM- or

the muscle-isolated $CD45^+/Sca1^+$ cells was employed to estimate more globally the ability of these cells to be reprogrammed and their relationship with the satellite cells. This analysis contributes to elucidate the role of the muscle microenviroment and the specific myogenic pathways activated, during this process. Lastly, *Pax7* was ectopically expressed in BM-$CD45^+/Sca1^+$ cells to examine the capacity of this transcription factor to induce these cells to acquire satellite-cell-like characteristics and to increase their myogenic potential *in vivo*.

3 Material and Methods

3.1 Reagents.

3.1.1 Chemicals.

All chemicals were obtained from FLUKA, Sigma Chemical Co. or Roche. Other chemicals used during the study were obtained from the following sources:

Protein markers	Biorad
X-Ray film	KODAK
Hydrolysed milk powder	Nestlè
Nitrocellulose for western blot	Protran, Schleicher and Schuell
Nylon membrane for Southern	Hybond-N, Amersham
Collagenase type II	Worthington
OCT	Tissue -Tek
paraformaldehyde	Electron Microscopy Sciences

Table 4. Chemicals Source.

3.1.2 Radiochemicals.

P dCTP (Amersham).

3.1.3 Solutions and buffers.

Buffer for flow cytometry analysis	PBS containing 1 % v/v FBS
Fixation/Permeabilization buffer	25% v/v Fixation/Permeabilization concentrate (eBiosciences) 75% v/v Fixation/Permeabilization diluent (eBiosciences)
Permeabilization buffer	10% v/v Permeabilization buffer 10x (eBiosciences) 90% H_2O
1x TBE for agarose gel	0.0089 M Tris Base 0.0089 M boric acid 0.0002M EDTA, ph8.0
1x TAE for agarose gel (for 1 litre)	0.002 M Tris Base 0.001 M Na Acetate 0.1mM EDTA, pH 8.0
100X Denhardts:	1g FICOLL 400 1g Polyvinylpropylene(PVP) 1g BSA (fraction V) Up to 50 ml with water
20X SSC	350.6g NaCl 176.5g Citric acid, trisodium salt DNase free distilled water up to 2 litres
20X SSCP	88.2g Sodium citrate (trisodium salt) 140.3g NaCl 43.7g Na_2HPO_4 12.7g $Na_2HPO_4.H_2O$ DNase free distilled water up to 1 litre
Southern Hybridisation solution (for 20 ml):	6 ml 20X SSC 0.2 ml 100X Denhardts 10 ml deionised formamide 1 ml 10% w/v SDS 2g Dextran Sulphate

	2.8 ml of B.S256+probe
B.S256(10X)	16 mg polyA 16 mg polyC 400 mg yeast t-RNA 100 mg Salmon Sperm DNA 9 mg *E. coli* DNA
Western blot Solution A	10mM HEPES pH7.9 1.5mM MgCl2 10mM KCl, 0.5mM DTT
Western blot Solution B	300mM HEPES pH7.9 1.4M KCl 30mM MgCl2
Western blot 5x loading buffer	300mM Tris Hcl pH 6,8 10% SDS 50% glycerol 25% beta-Mercaptoethanol 0.04% of Bromo Phenol Blue
Western blot Transfer buffer (for 1 litre):	3g Tris 14.4 g Glycine 200 ml Methanol 800 ml Water

Table 5. Solutions and buffers.

3.1.4 Restriction enzymes and bacterial strains.

All DNA modification enzymes, restriction enzymes and buffers were received from Roche or New England Biolabs. The DH5 bacterial strain (F', hsdR17, rk-mk+, recA1, endA1) was used for all transformations, and grown in Luria Broth media (LB, Becton Dickinson) or Terrific Broth (TB, Becton Dickinson).

3.1.5 Antibodies.

Isotype control antibodies, rat anti-mouse IgG (FITC, R-PE, PerCP or Tricolor conjugated)	PharMingen
Streptavidin comjugated with Tricolor	Caltag Laboratories
Anti-mouse CD16/CD32	PharMingen
Anti-mouse CD3-PE (clone 17A2)	PharMingen
Anti-mouse B220-PE and -APC (clone RA3-6B2)	PharMingen
Anti-mouse Sca1-PE (clone E13-161.7)	PharMingen
Anti-mouse Sca1-PE (clone E13-161.7)	PharMingen
Anti-mouse CD45.2-PerCP-Cy5.5 (clone 104)	PharMingen
Anti-mouse CD45.1-PE or -FITC (clone A20)	PharMingen
Anti-mouse CD11b-PE and -APC (clone M1/70)	PharMingen
Anti-mouse CD45-PerCP or –APC (clone 30-F11)	PharMingen
Anti-mouse CD117-APC (clone 2B8)	PharMingen
Anti-mouse IgG HRP-conjugated	DAKO Cytomation
Anti-rabbit IgG HRP-conjugated	DAKO Cytomation
Rabbit anti-GFP	Molecular Probes
Alexa Fluor 488 donkey anti-rabbit	Molecular Probes
Alexa Fluor 488 donkey anti-goat	Molecular Probes
Alexa Fluor 594 donkey anti-mouse	Molecular Probes
Goat anti-δgal	SantaCruZ
MHCf	Novocastra
DYS2	Novocastra
Anti-human/mouse Pax3-PE	R&D Systems
Anti-Pax7	DHSB
Anti-human histone H2B	Abcam

Table 6. Antibodies list.

3.2 Cell culture reagents.

3.2.1 Plastic ware.

All standard tissue culture plastic ware, (tissue culture flasks, roller bottles, collagen coated transwell apparatus, tissue culture plates) was obtained form Corning Costar.

3.2.2 Media.

IMDM	Biowhittaker
HAM's F-10	Sigma
RPMI 1640	Euroclone
BIT 9500	Stem Cell technology
DMEM	Biowhittaker

Table 7. Culture Media.

3.2.3 Sera, Supplements and antibiotics.

Fetal Bovine Serum	Euroclone
Trypsin	Gibco Brl
L-Glutamine	Gibco Brl
Penicillin/Streptomycin	Gibco Brl
HEPES	Gibco Brl
Polybrene	Sigma
Beta-mercaptoethanol	Sigma

Table 8. Sera, Supplements and antibiotics.

3.2.4 Growth factors.

Recombinant human interleukin-6 (rhIL-6)	R&D Systems
Recombinant human Flt-3 Ligand (Flt3-L)	Pepro Tech, England
Recombinant murine Stem cell factor (mSCF)	Pepro Tech, England
Recombinant murine interleukin-3 (mIL-3)	PeproTech, England
Recombinant murine basic Fibroblast growth Factor (bFGF)	Pepro Tech, England

Table 9. Growth factors.

3.2.5 Other.

Phosphate buffered Saline (PBS) was obtained from Euroclone.

3.3 Mice.

C57BL/6/Ly-5.2, C57BL/6-Tg(ACTbEGFP)1Osb (expressing *eGFP* constitutively) and C57BL/6/Ly-5.1 mice were purchased from Charles River laboratories (Calco, Milan, Italy) and Jackson laboratories (Bar Harbor, Me.), respectively. *Myf5*$^{nlacZ/+}$ [135] mice and MLC3F-*nlacZ*-E [136] mice, kindly provided by Dr. Tajbakhsh, and *Dmd*$^{mdx\text{-}4Cv}$ [137] mice were bred in a C57BL/6 genetic background. To obtain a GFP transgenic mouse in which the nuclear-localising beta-galactosidase is under the control of the MLC3F or the Myf5 promoter, C57BL/6-Tg(ACTbEGFP)1Osb heterozygotes were crossed with MLC3F-*nlacZ*-E homozygotes or *Myf5*$^{nlacZ/+}$ mice, respectively. *MyoD* null mutants [138] and *Pax7* heterozygote [139] adult mice were bred in a mixed C57BL/6/-129/Sv genetic background. *Pax7* heterozygotes were crossed with *Myf5*$^{nlacZ/+}$ mice or MLC3F-*nlacZ*-E mice, and in the F2 generation *Pax7*$^{-/-}$/*Myf5*$^{nlacZ/+}$ mice and *Pax7*$^{-/-}$/MLC3F-*nlacZ*-E double transgenic mice were obtained. All procedures were approved by the Animal Care and Use Committee of the Fondazione San Raffaele del Monte Tabor (Institutional Animal Care and Use Committee, licence number 299) and were communicated to the Ministry of Health and local authorities according to Italian law.

3.4 Methods.

3.4.1 Pax7d Cloning and plasmid construction.

Total RNA from proliferating myoblasts was extracted using the TRI REAGENT (SIGMA), following the supplier's instructions. cDNA was synthesised using the SuperScriptTM III First-Strand synthesis system for RT-PCR (INVITROGEN). RT-PCR for cloning of the Pax7d cDNA was performed with the following sets of primers: Pax7-1F (5'-GGATTCGTCTCCAGCGTGTGCAGAAAT-3'), Pax7-1R (5'-ACCCTAGTAGGCTTGTCCCGTTTCCA-3'). The Pax7d cDNA was cloned into the pCR2.1 TOPO TA vector (INVITROGEN) according to supplier's guidelines and it was partially sequenced to ensure that the Pax7d isoform was cloned. The resulting plasmid was digested with *BamHI* and was purified using the QIAquick PCR Purification Kit of QIAGEN. The linearised plasmid was digested with *XhoI* and the Pax7d cDNA was gel extracted using the QIAquick Gel Extraction Kit of QIAGEN. The lentiviral vector *PGK.GFP* [140] was digested with *BamHI* and *SalI* and the vector backbone was gel extracted. The Pax7d cDNA was inserted into the lentiviral vector by using the TAKARA DNA ligation kit Ver.1.

3.4.2 Polymerase chain reaction on genomic DNA.

DNA extraction from CFUs and WBM were performed, using the ChargeSwitch Forensic DNA Purification Kit of Invitrogen, according to supplier's instructions and as described in section 3.4.3, respectively. PCR reaction specific for the *MCK.GFP* vector was performed with the following set of primers: MCKF (5'-CTTTCCATGGCTGCTCGC-3') and MCKR (5'-CGTGGTCTTAGGCTCTGTAC-3'). For the PCR detection of the integrated *PGK.Pax7* provirus, the following primers were used: Pax7-2F (5'-TTGTGTCTCCAAGATTCTGTGCCGA-3') and Pax7-2R (5'-

GCCATACGGGAAGCAATAGCATGAT-3'). The dNTP's were obtained from Roche while the AmpliTaq gold polymerase enzyme, the PCR buffer and the $MgCl_2$ solution were obtained from Applied Biosystems. The PCR samples were mixed with DNA loading dye and run on 1.2% w/v agarose gels in TBE buffer, containing Ethidium Bromide, and visualised on a UV lamp.

3.4.3 Southern blot analysis.

Genomic DNA was isolated from 1–5x10^6 cells using the QIAGEN QIAmp DNA mini Kit, following the manufacturers instructions. Nucleic acid concentration was determined using the 260/280nm absorbance measurements on a spectrophotometer.

For the creation of the probe, a 738bp DNA fragment corresponding to the RRE region, was obtained by digesting 20 g pRRL.sin-18. CMV.GFP.WPRE lentivector with *AvaI* and *MunI*. After running on a 1% w/v agarose gel, in TBE buffer, the band corresponding to the required DNA fragment was excised using a scalpel, and it was purified using the QiaexII DNA extraction kit from QIAGEN following the manufacturer's instructions. The concentration was measured and the probe was labelled as described below.

10 g of the genomic DNA was digested with *AflII* concentrated enzyme over 16–18 hours. The digested DNA was run on a 0.8% agarose gel in TAE buffer, containing ethidium bromide, overnight at 35 volts. The gel was photographed.

The DNA was denatured by incubating the gel in 0.2M NaOH, 0.6M NaCl for 1 hour with one exchange of the buffer. The gel was then neutralised by incubation for 1 hour in 1 M Tris HCl, pH7.5, 1.5M NaCl. Southern blot assembly was performed as described by Maniatis and colleagues [141] using Hybond Nylon filters from Amersham

(presoaked in distilled water and 10X SSC) overnight in 10X SSC. After blotting the filter was rinsed in 6X SSC and cross-linked using a Stratalinker.

The radioactive probe was mixed with B.S256, boiled for 10 minutes and quenched on ice, prior to adding to the hybridisation solution. This was performed to ensure that the denaturation of the probe.

The filter was washed with 0.1X SSC, 0.5% w/v SDS at 65^0C for 1 hour, exchanging the buffer twice, followed by an overnight incubation in pre-hybridisation solution at 42^0C.

Pre-hybridisation solution:

5X Denhardt's
5X SSCP
50% deionised formamide

The pre-hybridisation solution was removed and replaced with hybridisation solution containing the radioactive labelled probe (2 x 106 dpm per ml). The filter was incubated overnight at 42^0C, followed by a 30-minute wash in 2X SSC, 0.1% SDS (with one change) at room temperature and an one-hour wash at 55^0C with 0.1X SSC, 0.1% w/v SDS (with 2 changes). It was subsequently dried and exposed to KODAK autoradiograph film for various times and the films developed.

Radioactive labelling was performed using the Roche Random primed DNA labelling kit and the quick spin columns (to eliminate the unincorporated nucleotides), following the manufacturer's instructions.

3.4.4 Poliacrylamide gel electrophoresis (PAGE) and western blotting.

15% denaturating PAGE gels were made following the published instructions (Maniatis, Fritsch et al, 1982), and were assembled on the Biorad miniprotean II gel apparatus following the manufacturer's recommended instructions. 1–5x10^6 cells were lysed in 100 l of solution A for 10 minutes on ice. 3 µl Triton X-100 10% were added

and the samples were centrifuged at 11.000 rpm for 1 minute at 4°C. The supernatant was collected and 11μl of solution B were added. The samples were centrifuged at 11.000 rpm for 1 minute at 4°C. The supernatant was collected and the protein concentration was determined using the BIORAD Protein Assay solution, following the instructions recommended by the manufacturer. Twenty illigrams of the lysate were mixed with 5 l of 5-fold concentrated loading buffer, containing beta-mercaptoethanol, and the resulting mixture was loaded into the well of the PAGE gel. The gel was run in Tris-Glycine buffer (27.9 g glycine, 6 g Tris, dissolved in 1 litre of water), containing 0.1% v/v SDS, at 40V, until the Bromo Phenol Blue dye had run out of the gel. As a molecular weight marker, 4 l of the Rainbow marker (Biorad) were used. Nitrocellulose membranes (Scleicher and Schuell) cut to the same size as the PAGE gel were pre-soaked in transfer buffer.

Blotting was performed using the Biorad Semidry Transfer Cell, assembled following the manufacturer's instructions. Upon assembly, the transfer was performed for 30 minutes at 15 volts at room temperature.

After the proteins had been transferred, the membrane was blocked in PBS containing hydrolysed milk (5% w/v) for 60 minutes. The anti-Pax7 (dilution: 1/1000, DHSB) and the anti-H2B (dilution: 1/20000, Abcam) antibodies (sc-21757, Santa Cruz Biotechnology) were added to the blocking reaction incubated with agitation for 1 hour at room temperature. The membrane was washed three times with PBS/0.05% v/v Tween 20, and fresh blocking solution containing the polyclonal goat anti-mouse IgG Horseradish Peroxidase (HRP)-conjugated antibody (DAKO Cytomation) and the polyclonal goat anti-rabbit IgG Horseradish Peroxidase (HRP)-conjugated antibody (DAKO Cytomation) at a 1 in 5000 dilution were added to the membrane. Incubation was performed for one hour at room temperature with agitation. The membrane was washed as before and the peroxidase activity was measured using the commercially

available ECL kit (Amersham) following the recommended instructions. The membrane was exposed to KODAK autoradiograph film and it was developed.

3.4.5 Transformation of bacteria and bacterial and plasmid DNA preparations.

DNA to be transformed was mixed with 100 l of competent DH5 cells prepared specifically for the heat shock mediated transformation method **(Maniatis, Fritsch et al, 1982)**. The mix was incubated on ice for 20 minutes, then 1 minute and 45 seconds in a waterbath at 42°C, and then returned to the ice for further 10 minutes. The mix was then added to 1 ml of LB media and incubated at 37°C with agitation for one hour. The bacteria were then plated onto LB agar plates, containing the required antibiotic resistance and were placed overnight in a 37°C incubator. The following day, colonies were picked and placed into 1.5 ml of LB media containing antibiotics, and were grown overnight with agitation at 37°C. The small bacteria preparations were used for small or large-scale DNA preparation as required.

Small amounts of plasmid DNA were purified from bacteria, for restriction analysis, using the GenElute Plasmid Miniprep kit (Sigma). We isolated plasmid DNA using the High purity Plasmid Megaprep system kit from Marlingen following the supplier's instructions.

3.4.6 Generation of Lentiviral vectors.

Viral stocks pseudotyped with the vesicular stomatitis G protein (VSV-G) were prepared by transient co-transfection of 293T cells using a three-plasmid system (the transfer vector, the pCMVμR8.74, encoding for *Gag*, *Pol*, *Tat* and *Rev*, and the pMD.G plasmid encoding for VSV-G). Moreover, a fourth plasmid for the synthesis of the Rev

protein under the control of RSV promoter (pRSV.Rev) was added in order to increase the level of the unspliced viral RNA to be packaged.

Transfection of DNA using Calcium phosphate precipitation:

1. $9x10^6$ 293T cells were seeded in a 15 cm dish, approximately 24 hours before transfection in IMDM, 10%FBS, Penicillin (25U/ml), Streptomycin (25U/ml).
2. The medium was changed 2 hours before transfection.
3. The plasmid DNA mix was prepared by adding 7 g ENV plasmid (pMD2-VSV-G), 16.25 g of Core Packaging plasmid (pCMVμR8.74), 6.25 g Rev-expressing plasmid (pRSV.Rev) and 25 g transfer vector per dish. The plasmid solution was made up to a final volume of 2.25 ml with 0.1XTE/H_2O (2:1) in a 50 ml polypropilene tube. Finally 250 l of 2.5M $CaCl_2$ were added to the mix.
4. The precipitate was formed by drop-wise addition of 2.5 ml HBS (281 mM NaCl, 100 mM HEPES, 1.5mM Na_2HPO_4) to the DNA-TE-$CaCl_2$ mixture while vortexing at full speed. The precipitate was then added to the 293T cells immediately. High magnification microscopy of cells revealed a very small granular precipitate of the CaPi-precipitate plasmid DNA, initially above the cell monolayer, and after incubation in a 37°C incubator overnight on the bottom of the plate in the spaces among the cells.
5. The CaPi-precipitated plasmid DNA was allowed to stay on the cells for 14-16 hours, after which the media was replaced with fresh media. The medium was discarded as infectious waste.
6. The cell supernatant was collected at 24 hours after the media exchange.
7. The cell supernatant was centrifuged at 1500 rpm, for 5 minutes and filtered through a 0.22 m pore nitrocellulose filter.
8. The conditioned supernatant was concentrated (see section 3.4.7).

3.4.7 Viral concentration.

1. The virus-containing supernatant was concentrated by ultra-centrifugation at 50,000 x g (19,500 rpm in SW28 rotor) 2 h at RT.
2. The supernatant was discarded by decanting
3. The final pellets were re-suspended in a very small volume (1/350 of the starting volume of medium) of sterile PBS.
4. The aliquots were pooled in a small tube and rotated on a wheel at 4°C for 1 h (10 rpm).
5. The virus was divided into small aliquots (20-50 µl) and stored at -80°C and the viral titre was determined after freezing.

3.4.8 Titration of Lentiviral vectors.

Serial ten-fold dilutions of the viral stocks were prepared (from 10^{-3} to 10^{-8}). 2x105 HEL cells were re-suspended in 1 ml of each serial dilution and plated in a well of a 24-well plate. Polybrene was added to each well (final concentration: 8µg/ml). The infected HEL cells were incubated for 24 hours at 37°C in a 5% CO_2 incubator and the day after transduction the cells were washed with 1 ml of PBS and resuspended in 1 ml of fresh medium.

For the *GFP*- and the *Pax7*-expressing vectors, on day 5 after the transduction the cells were collected in flow cytometry tubes, washed with 2 ml of PBS and centrifuged at 1500 rpm for 5 min at RT. The supernatant was aspirated and the pellets were re-suspended in 1 ml of PBS 1% FBS, FACS analysis for *GFP* or *Pax7* expression was performed and the titre was calculated as follows:

$$\text{TU/ml} = \frac{(\%\ \text{of GFP}^+\ \text{cells})/100 \times \text{number of plated cells (2x105)}}{\text{Transduction volume} \times \text{viral vector dilution}}$$

TU=transducing unit.

For the *MCK.GFP* vector, on day 20 after transduction, the infected cells were collected, their DNA was extracted and the transduction efficiency was evaluated by quantitative real-time polymerase chain reaction (qPCR) as described in sections 3.4.3 and 3.4.9, respectively. The titre was calculated as described above for the *GFP*- and the *Pax7*-expressing vectors.

Note: The MOI (multiplicity of infection) is defined as the fraction: Transducing units / number of cells to be infected.

3.4.9 Maintenance and transduction of stable cell lines.

Human erythroleukemic (HEL) cells were maintained in RPMI-1640 with 10% FBS. The 293T cell line was maintained in IMDM media and 10% FBS. All these media were supplemented with penicillin/streptomycin at 100 units/ml, and L-glutamine [2 mM]. The adherent cells (293T) were typically passaged at 1/5 to 1/20 dilutions (depending on experimental requirements.) twice a week by washing once in PBS, adding trypsin solution to the adherent cells, incubating for 3 minutes and then re-suspending in fresh media. The non-adherent cells (HEL) were passaged by simple dilution of the cells in fresh media (usually at 1/10 dilutions twice a week). The HEL and the 293T cell lines were transduced with viral supernatants at different MOI in the presence of 8 μg/ml of polybrene. All cells were maintained at 37^0C in a humidified incubator with atmospheric 5% CO_2.

3.4.10 Quantitative PCR.

The average vector copy number per cell was measured by quantitative real-time polymerase chain reaction (qPCR). PCR was performed using primers and probe that anneal in the RRE sequence: RRE forward primer (5'-TGAAAGCGAAAGGGAAACCA-3'), RRE reverse primer (5'-

CCGTGCGCGCTTCAG-3') and RRE probe (5'-VIC-AGC TCT CTC GAC GCA GGA CTC GGC-MGB-3') (Applied Biosystems). Primers and probe complementary to the human *GAPDH* gene were used to normalize for the DNA content. The GAPDH primers were as follows: GAPDH forward primer (5'-ACCACAGTCCATGCCATCACT-3'), GAPDH reverse primer (5'-GGCCATCACGCCACAGCTT-3') and GAPDH probe (5'-TET-CCACCCAGAAGACTG TGGATGGCC-MGB-3') (Applied Biosystems). MasterMix containing dNTPs, polymerase and buffer, was obtained from Applied Biosystems. The quantitative PCR was performed in a ABI 7900 Real Time PCR machine (Applied Biosystems).

3.4.11 Primary myoblast cultures and co-cultures.

Purified muscle cells were cultured (10^5 cells/ml) in HAM's F-10 medium (Sigma) supplemented with 20% FBS and 5ng/ml bFGF (Peprotech), on collagen-coated dishes, after pre-plating for 1 hour in uncoated dishes. BM cells were isolated as described below and were cultured for 2 days in serum-free IMDM, containing BIT serum substitute, 50 ng/ml muSCF, 10 ng/ml muIL-3, 10 ng/ml huFlt-3L and 20 ng/ml huIL-6. The cultured BM cells or BM-derived cells sorted from the muscle for *CD45* and *Sca1* expression were mixed with proliferating primary myoblasts at a ratio 1:1 and were seeded on collagen-coated wells of 6-well plates. The cell density was 4 x 10^4 cells/well. The co-cultures proliferated in myoblasts proliferation media for 3–4 days and differentiated in DMEM supplemented with 5% horse serum for 2–3 days. For the sorted BM cells, $CD45^+$ cells from the $Myf5^{nlacZ/+}$ mice were put together with $CD45^-$ cells from the WT mice in their ratio in the whole BM. This process was repeated for the $CD45^-$, $Sca1^+$, $Sca1^-$, c-kit^+ and c-kit^- cells and the diverse sub-populations were subsequently co-cultured with myoblasts.

3.4.12X-gal staining and immunofluorescence.

For the X-Gal staining, the cultured cells were fixed with 0.2% glutaraldehyde and were incubated overnight at 37°C with 5-bromo-4-chloro-3-indolyl δ-D-galactoside solution. For the enumeration of regenerated myofibers, the TA muscles were isolated, fixed in 4% paraformaldehyde, cryoprotected in sucrose 5–20%, embedded in OCT and frozen in isopentane cooled in liquid nitrogen. The immunofluorescence staining was performed on 10- to 16-μm cryosections. In the case of cultured cells, the fixation and the permeabilization were performed with 2% paraformaldehyde and 3% sucrose for 10 minutes and 0.5% (v/v) Triton-X for 10 minutes, respectively. Subsequently, the samples were blocked for 1 hour with donkey serum and then incubated with the following primary antibodies: rabbit anti-GFP (Molecular Probes, 1/200), goat anti-δgal (SantaCruZ, 1/50), mouse anti-Pax7 (DHSB, 1/1000) and MHCf (NOVOCASTRA, 1/40) for 1hour at room temperature. Alexa Fluor 488 donkey anti-rabbit, Alexa Fluor 488 donkey anti-goat and Alexa Fluor 594 donkey anti-mouse (Molecular Probes, 1/500) were used for the secondary detection (45 minutes at room temperature) and Hoechst 33342 dye for the nucleus visualization. To rule out the possibility of auto-fluorescence mistaken for GFP, only myofibers positive for a length of more than 150-μm were considered.

3.4.13Transduction of murine BM cells.

Murine BM cells were harvested from mice as described below and infected for 18 hours with viral stocks at an MOI of 100 or 50 in serum-free IMDM, containing BIT serum substitute, 50 ng/ml muSCF, 10 ng/ml muIL-3, 10 ng/ml huFlt-3L and 20 ng/ml huIL-6. The transduced BM cells were washed with PBS and plated at a density of 0.2 x 10^5 cells/ml in methylcellulose medium that supports the growth of murine hamatopoietic colonies (see section 3.4.14). For the GFP-expressing vectors, the

transduced BM cells were also grown in liquid culture in the same medium described above and were analysed by flow cytometry for GFP expression on day 5 after the transduction. For the *MCK.GFP* and the *Pax7*-expressing vectors, single colonies were isolated 10–14 days after plating and the transduction efficiency was assessed by PCR screening of CFCs using primers specific for each vector (see section 3.4.1).

3.4.14 CFC assay.

BM and muscle-derived cells, purified as described below, were washed with PBS and plated at a density of 0.2 x 10^5 and 10^5 cells/ml, respectively, in methylcellulose medium that supports the growth of murine colonies, containing rmSCF, rmIL3, hIL6 and hrEPO (M3434, Stem Cell Technologies). For the assays of BM and muscle-derived cells, the colony number was assessed at day 10 and 8 after plating, respectively. For the lentivirally transduced BM cells, the single colonies were isolated 10–14 days after plating and the transduction efficiency was assessed by PCR screening of CFCs using primers specific for the vector (see section 3.4.2).

3.4.15 Flow cytometry and cell sorting.

Muscle and BM cells were prepared as described below, resuspended in IMDM, supplemented with 10% FBS, at a density of 10^7 cells/ml and incubated with anti-CD16 antibody for 15 minutes at 4°C (Pharmingen, San Diego, CA) to block nonspecific binding to the Fc receptor. The purified cells were subsequently stained with the appropriate antibody (Table 6) for 45 minutes at 4°C. The unbound antibodies were removed by a final wash with PBS before the analysis on FACScan or FACSCalibur (Becton Dickinson, Mountain View, Calif.). The cells were sorted by flow cytometry by a double laser instrument (FACSVantage, BecktonDickinson) and the purity was close to 95%. The chimaerism in the bone marrow and the muscle was determined by staining

with anti-CD45.1 and anti-CD45.2 antibodies and it was calculated from the CD45.2/(CD45.1+CD45.2) ratio cell counts. All the BM-transplanted mice used, reached a chimaerism higher than 85%.

For the Pax7 intranuclear staining, the cells previously stained for the surface markers as described above were fixed and permeabilized with the Fixation/Permeabilisation solution of eBiosciences overnight at 4°C. The cells were subsequently re-suspened in Permeabilization buffer (eBiosciences) at a density of 10^7 cells/ml, re-incubated with the anti-CD16 antibody and finally stained with an anti-human/mouse Pax3-PE antibody (R&D Systems), able to cross-react also with Pax7, for 45 minutes at room temperature.

3.4.16 Magnetic enrichment of BM-Sca1$^+$ cells.

Murine Sca1$^+$ cells were purified from the total BM by staining the BM cells with PE-conjugated anti-mouse Sca1 antibody. The cells were washed with PBS (BSA: 1% w/v) to remove the unbound antibodies and were magnetically labelled with anti-PE Microbeads (Milthenyi Biotech, Auburn, Calif.). Then the cells were loaded on a MS column and separated using the Mini MACS separator (Milthenyi Biotech, Auburn, Calif.). Both the positive and negative fractions were FACS analyzed to check the purity.

3.4.17 Sacrifice of mice and BM and muscle cells isolation.

Mice were sacrificed by carbon monoxide inhalation and their femurs and tibias were removed by surgical procedure, the BM was obtained by flushing the bones with IMDM with 1% FBS using a syringe and a 22-gauge needle. After re-suspension, the cells were passed through a 40-μm cell strainer. The cell numbers were counted and the viability was assessed by trypan blue staining. For the isolation of muscle cells, the

tibialis anterior and the quadriceps muscles were excised from transplanted mice. The bones and the tendons were removed and the muscle tissue was thoroughly minced and then digested at 37°C with 0.2% collagenase type II-filtered (Worthington) for 30 minutes. The tissue was triturated briefly by using a 10-ml pipette and then passed successively through a 100-μm, a 70-μm and a 40-μm cell strainer (Falcon). The cells were collected by centrifugation, re-suspended in the appropriate medium and counted using a haemocytometer.

3.4.18BM transplantations.

For BM transplantation experiments, six- to eight-week-old mice were used as donors or recipients. 2–3 million of BM cells were prepared as described above, washed twice in PBS, ensured to be a single cell suspension and suspended in 100 μl of physiological serum. The cells were taken up into a 1 ml syringe with low gauge needle (26Gx 1/2") and transplanted via intravenous injection into six-week-old C57BL/6 or *Pax7*$^{-/-}$ recipients that had received 11 or 10 Gy of irradiation, respectively. Tail vein injection was performed, holding a high intensity lamp to the tail of the mouse and waiting for the veins to become clearly visible. The needle was then gently inserted into the visible vein and the plunger very slowly depressed to inject the cells into the mouse. After successful completion of injection the mice were returned to their cages and neomycin antibiotic was included in the water supply to prevent bacterial infections.

3.4.19Muscle injury and isolation.

30 μl of cardiotoxin [0.1 mM] (Sigma) were injected into the TA and the quadriceps muscles using a 29G syringe. Mice were sacrificed from 4 days to 5 weeks after injury and the TA muscles were cryopreserved and used for immunofluorescence, or muscle-cell preparations were prepared as described above. The cells were counted

using a haemocytometer, collected by centrifugation and re-suspended in the appropriate medium. They were subsequently used for flow cytometry sorting and/or culture, and X-gal staining or immunofluorescence imaging followed.

3.4.20 Microscopy, statistics and bioinformatics.

All the samples were visualised using a 1X50 Olympus inverted microscope at room temperature and the pictures were acquired with a DS-L1 Nikon camera and processed with Adobe Photoshop CS version 8. All the statistical analyses were two-tailed tests and performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, USA). The frequencies and the errors represent mean values and errors of the means, respectively, unless otherwise stated. The gene expression data were analysed with the online Ingenuity Pathway Analysis system (Ingenuity Systems, www.ingenuity.com) using the Ingenuity Pathway Knowledge Base as the dataset reference.

3.4.21 DNA microarray analysis.

$CD45^+/Sca1^+$ cells isolated from the BM or the muscle were processed fresh and their RNA was extracted and treated with DNase 1, using the RNAqueous®-4PCR kit (Ambion) according to the manufacturer's instructions. The DNA microarray analysis was performed using the Affymetrix MOE430A Gene Chip arrays as previously described [142].

4 Results

4.1 BM-CD45$^+$/Sca1$^+$ cells undergo myogenic specification, differentiation and incorporation into muscle fibers in a Pax7-independent manner.

4.1.1 BM cells enriched for HSC surface markers are able to undergo myogenic specification, differentiate and contribute to myotube formation.

We set up a co-culture assay of BM cells and primary myoblasts to investigate the molecular mechanism underlying in BM-to-muscle transition and to assess the ability of BM cells to express muscle-specific genes, such as *Myf5*. Myf5 is an excellent marker of myogenic specification (see section 1.1.2.1), therefore we decided to employ a knock-in mouse (*Myf5$^{nlacZ/+}$*), which produces the nuclear-localising δ-galactosidase (δ-gal) under the control of the Myf5 promoter [135]. BM cells derived from *Myf5$^{nlacZ/+}$* mice were co-cultured with unlabelled primary myoblasts. After 3–4 days of proliferation, X-gal staining was performed and 68 ± 24 (n=4) δ-gal$^+$ cells were detected from 5 x 10^4 initially plated BM cells (0.13% ± 0,05%, n=4, Figure 10a and 10b). This observation showed that certain BM cells are able to express *Myf5*, following co-culture with myoblasts. However, they never gave rise to myogenic clones, suggesting that these cells cannot proliferate or self-renew like *Myf5*-expressing satellite-derived myoblasts do.

To ensure that the Myf5$^+$ BM cells are derived from haematopoietic progenitors, BM cells were sorted for the HSC surface markers CD45, Sca1, and c-kit. BM cells

from *Myf5*$^{nlacZ/+}$ and WT mice were sorted and subsequently combined in order to reconstitute the composition of WBM (see section 3.4.11). X-gal staining revealed that the cells sorted for CD45, Sca1, and c-kit expressed *Myf5* with a statistically significantly higher frequency than WBM (Friedman test: $p<0.0001$); while hardly any δ-gal^{+} cells were also found in the negative fractions at levels bellow the expected background of sorting cross-contamination (Figure 10c). These results demonstrate that BM cells enriched for HSC-surface markers can express the myogenic transcription factor *Myf5* in co-culture with myoblasts.

To further verify the myogenic specification of BM-derived haematopoietic cells, we set out to demonstrate the expression of other muscle-specific genes. Therefore, we repeated the co-culture experiments using BM cells derived from a double transgenic mouse, in which the nuclear-localising δ-galactosidase is under the control of the MLC3F promoter and *GFP* is ubiquitously expressed (see section 3.3). MLC is an early muscle differentiation marker and previously has been reported its expression both before and after the formation of myotubes by myoblasts. This feature allowed us to monitor the expression of *MLC* at different stages of the co-culture. After differentiation and X-gal staining, single *MLC*-expressing (δ-gal^{+}) cells were found (0.11% ± 0.033%, n=8) demonstrating that BM cells can also express *MLC* as a myogenic marker in addition to *Myf5* (Figure 11a and 11b). Interestingly, we also detected rare GFP^{+} myotubes (Figure 11c) containing δ-gal^{+} nuclei (Figure 11d), demonstrating that these BM cells can contribute to myotube formation (approximate frequency 0.01%) and that their nuclei continue to express *MLC* after fusion.

Furthermore, we decided to evaluate if the BM-derived haematopoietic cells expressing *Myf5* during the proliferation phase were likely the same cells that finally express *MLC* and differentiate. To this end, we exploited the known persistence of *Myf5* expression from myoblast to myotube [53] in a co-culture assay with BM of *Myf5*$^{nlacZ/+}$

mice. After spontaneous differentiation (i.e. no serum deprivation), immunofluorescence for the early myogenic differentiation marker MHCf and δ-gal revealed that certain BM-cells co-express *MHC* and *Myf5* (Figure 12 a–d). Moreover, *Myf5*-expressing myocytes and myotubes were also found (Figure 12f and 12e). Notably, these BM cells expressed the above myogenic markers only in co-culture with myoblasts and never in culture alone, indicating that interaction with muscle cells is required. Overall, these findings strongly suggest that HSC-derived myogenic progenitors do not only undergo myogenic specification (*Myf5* expression) but also differentiate (*MLC* and *MHC* expression) and contribute to the myotube formation *in vitro* (although at a much lower frequency than the first two events).

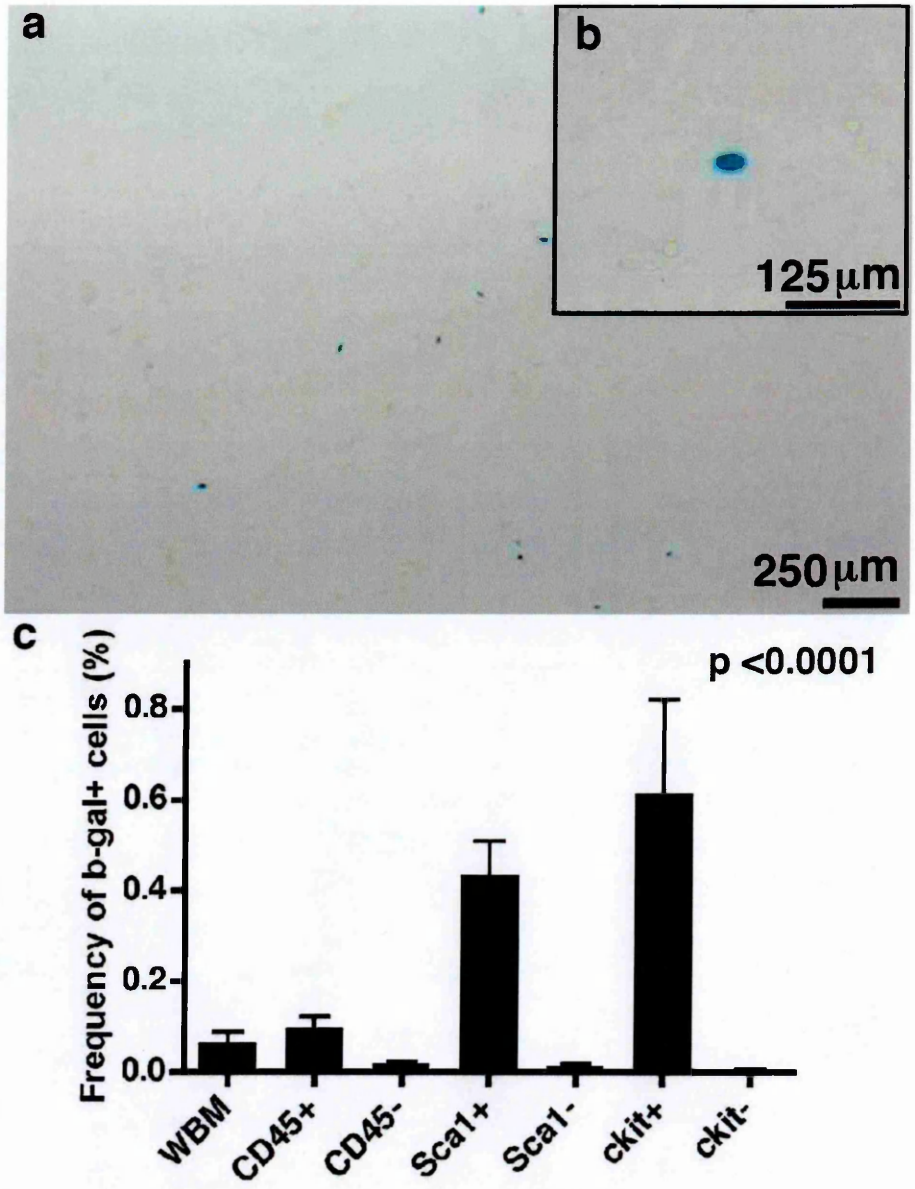

Figure 10. BM cells enriched for HSC surface markers occasionally express *Myf5* in co-culture with myoblasts.

(a–b) BM cells isolated from $Myf5^{nlacZ/+}$ mice were co-cultured with unlabelled primary myoblasts. After 4 days of proliferation, X-gal staining and bright field microscopy revealed several δ-gal^{+} (*Myf5*-expressing) cells (0.13% ± 0.05%, n=4). **(c)** Different BM sub-populations were co-cultured with myoblasts demonstrating that BM cells enriched for HSC markers express *Myf5* at a higher frequency than WBM. The error bars represent the standard error of the means of six different experiments and the p value was calculated using a Friedman test.

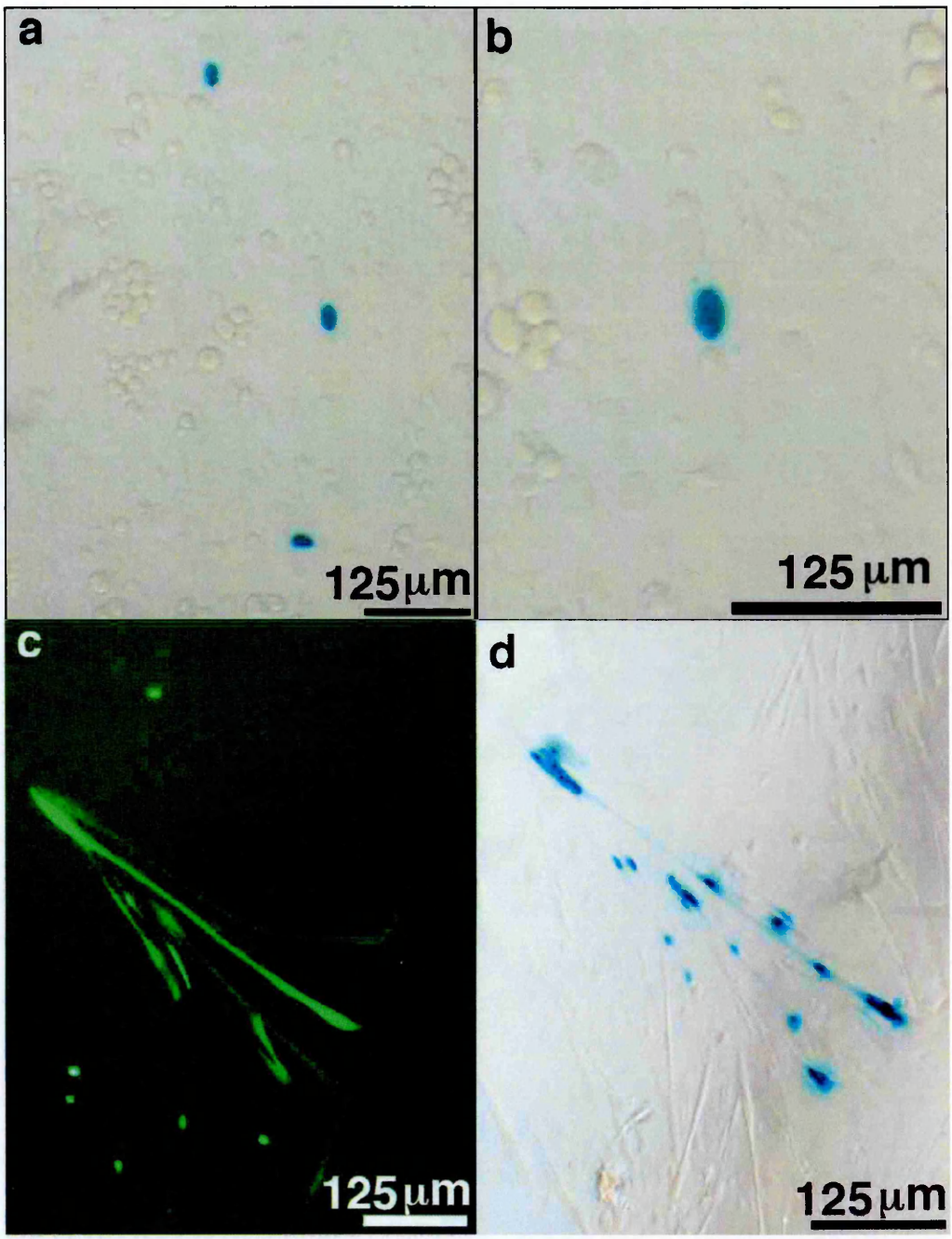

Figure 11. BM cells can differentiate and contribute to myotubes formation in co-culture with myoblasts.

(a–b) BM cells isolated from *GFP*/MLC3F-*nlacZ*-E mice were co-cultured with unlabelled primary myoblasts. After differentiation, X-gal staining and bright field microscopy revealed few δ-gal^{+} (*MLC*-expressing, differentiated) cells (0.11% ± 0.033, n=8). **(c–d)** GFP^{+} myotubes (panel c: fluorescence microscopy) were also detected synthesizing nuclear-localized beta-galactosidase (panel d: bright field miscroscopy).

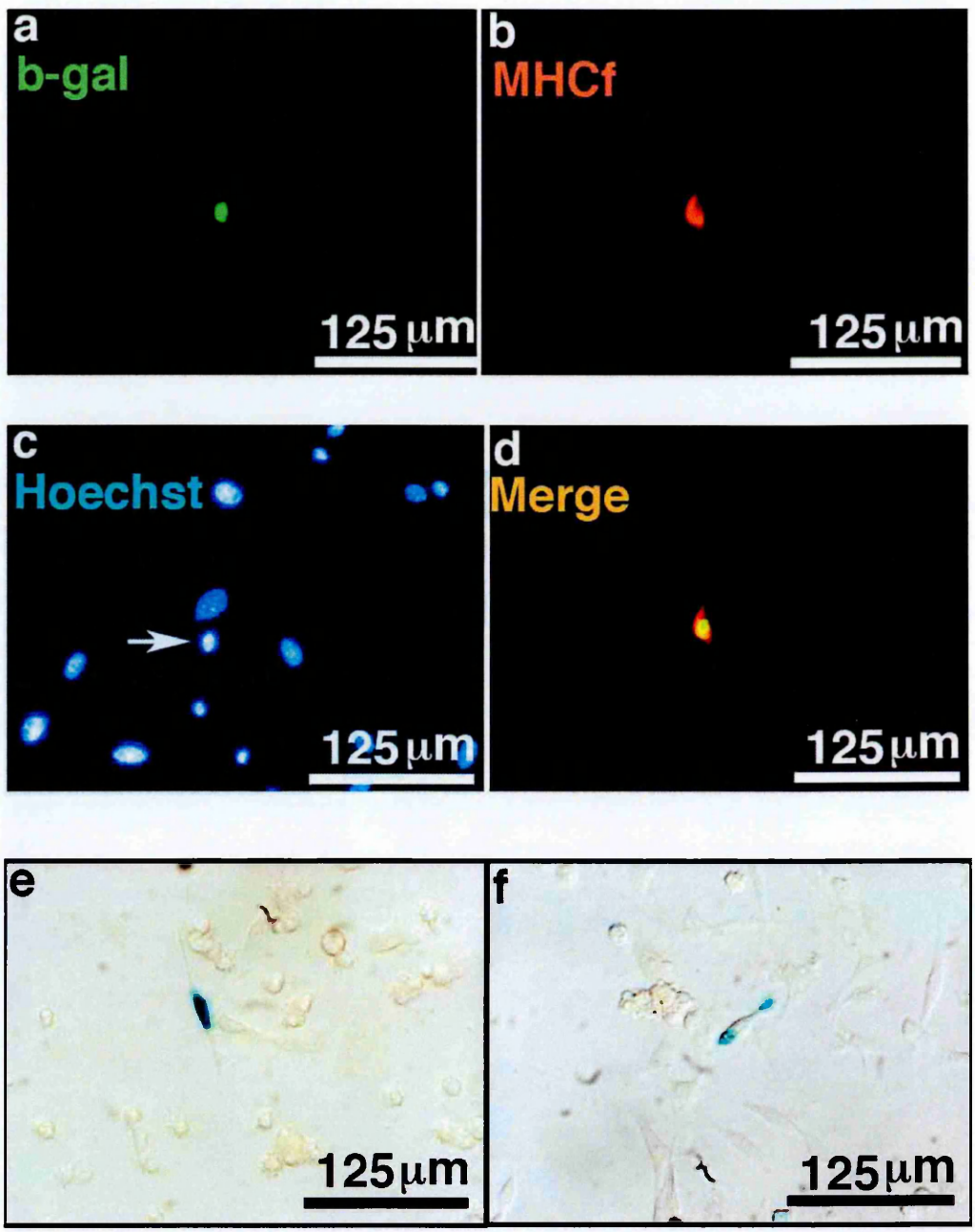

Figure 12. Step-wise myogenic differentiation of BM-derived myogenic progenitors in co-culture with myoblasts.

BM cells isolated from *Myf5*$^{nlacZ/+}$ mice were co-cultured with unlabelled primary myoblasts. **(a–c)** After spontaneous differentiation, the MHCf and b-gal immunofluorescence revealed *MHCf* and *Myf5* co-expression (A: b-gal, B: MHCf, C: Hoechst and D: electronic merge). **(f–e)** X-gal staining and bright field microscopy allowed the visualization of δ-gal^{+} (*Myf5*-expressing) myocytes (f) and myotubes (e).

4.1.2 BM-derived CD45$^+$/Sca1$^+$ cells isolated from regenerating muscle can be specified to myogenesis.

In order to demonstrate the capacity of BM-CD45$^+$/Sca1$^+$ cells for myogenic specification *in vivo*, *Myf5*$^{nlacZ/+}$/C57BL/6-*CD45.2* mice expressing *GFP* constitutively were used as donors for BM transplantation in lethally irradiated C57BL/6-*CD45.1* mice (see section 3.4.18). This CD45 isoform mismatch allowed us to distinguish between the haematopoietic cells derived from the donor and the residual recipient cells in chimeric mice, in which the bone marrow is *GFP/Myf5*$^{nlacZ/+}$ while the rest of the tissues are not labelled. After complete reconstitution of the haematopoietic system (six weeks), the right quadriceps and the tibialis anterior (TA) muscles were injected with cardiotoxin (ctx) to induce muscle regeneration. Four days after ctx injection, the mice were sacrificed and their injured muscles were removed and prepared for sorting by flow cytometry (Figure 13a). GFP$^+$ (BM-derived), CD45$^+$/Sca1$^+$ (haematopoietic) cells were sorted and co-cultured with unlabelled myoblasts and once again *Myf5*-expressing (δ-gal$^+$) cells were detected (0.02%± SD=0.008%, n=3), using X-gal staining (Figure 13b). Hence, certain BM-derived haematopoietic (CD45$^+$/Sca1$^+$) cells isolated from injured muscle can also express *Myf5*, as it had been initially shown for haematopoietic cells isolated from BM.

Additionally 3–5 weeks after injury, GFP$^+$ myofibers (52 ± 10, n=6) were observed in TA muscles of transplanted mice (Figure 13c). However, this result did not unequivocally demonstrate the hematopoietic origin of the BM cells that incorporate into muscle fibres, since the experiments were performed by transplanting WBM. To address this issue, we isolated by cell sorting the CD45$^+$ and the CD45$^-$ fractions of the BM derived from C57BL/6-*GFP* and C57BL/6 mice. Lethally irradiated C57BL/6-*CD45.1* mice were transplanted with either GFP$^+$/CD45.2$^+$ and CD45.2$^-$/GFP$^-$ cells or

$GFP^+/CD45.2^-$ and $CD45.2^+/GFP^-$ cells in proportions identical to the total BM. Six weeks after BM transplantation, TA muscles of transplanted animals were subjected to ctx injury and 3 weeks later were analyzed. Several GFP^+ myofibers were scored in TA muscles of mice transplanted with the $CD45^+/GFP^+$ cells (n=6), whereas no GFP^+ myofibers were found in the TA muscles of the mice transplanted with the $CD45^-/GFP^+$ cells (n=6), confirming the notion that only the cells from the haematopoietic compartment of the donor BM are able to participate to muscle regeneration, following BM transplantation.

To evaluate the contribution of donor-derived cells to the haematopoietic progenitors seeded in muscle, we further transplanted lethally irradiated C57BL/6-*CD45.1* mice (n=18, from 3 independent experiments) with BM cells isolated from transgenic C57BL/6-*CD45.2-GFP* donors. Six weeks after BM transplantation, a clonogenic assay of 10^5 cells derived from the muscle of the recipient mice revealed a mean of 12 ± SD=2 (n=3) GFP^+ CFUs (colony forming units) out of 14 ± SD=2 (n=3) $CFUs/10^5$ cells. These results reflect the high level of donor chimaerism, also shown by flow cytometry analysis, and suggest a BM origin for the muscle-resident haematopoietic cells in general. Additionally, the lethal irradiation used for BM transplantation depleted the ms-$CD45^+/Sca1^+$ cells (Figure 14a) and the donor BM-derived cells replenished this population after BM transplantation (Figure 13a and Figure 14b), further supporting this hypothesis. Morphological analysis showed that the donor-derived haematopoietic cells present in the muscle give rise to all the different types of haematopoietic colonies (e.g. Figure 13e), although the frequency was a tenth of that observed in CFU assays of progenitors isolated from WBM.

In order to assess whether the haematopoietic potential of BM cells, shown in healthy muscle, would be affected by the muscle injury; we transplanted lethally irradiated C57BL/6-*CD45.1* mice (n=35 from 3 independent experiments) with BM

cells from C57BL/6-*GFP* donors and induced muscle regeneration by ctx injection. When we plated in methylcellulose 10^5 mononucleated cells from the injured muscles, no typical haematopoietic colonies were observed. Both host- and donor-derived (GFP^+) cells gave rise to mixed colonies, containing both round- and fibroblast-shaped cells (Figure 13d). Similar results were obtained by plating $2x10^4$ sorted $GFP^+/CD45^+/Sca1^+$ cells in the methylcellulose media. These data show that the haematopoietic potential of the BM-derived cells while maintained in healthy muscle, it is highly impaired from injury, suggesting a change of cell fate during muscle regeneration.

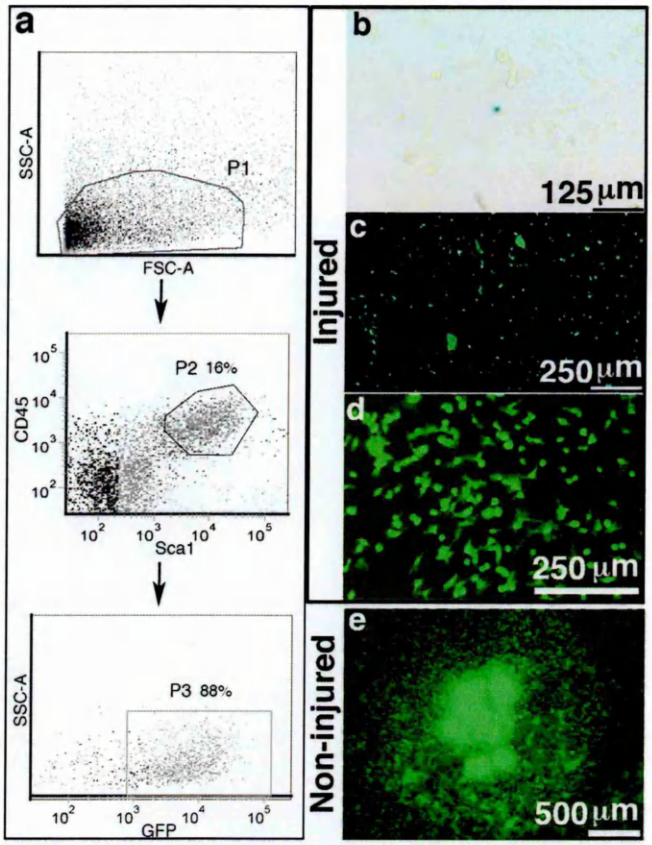

Figure 13. BM-CD45$^+$/Sca1$^+$ cells isolated from regenerating muscle can be specified to myogenesis.

(a) Flow cytometry analysis of injured muscles from mice transplanted with *GFP/Myf5$^{nlacZ/+}$* donor cells (top panel: forward scatter and side scatter; middle panel: *CD45* and *Sca1* expression; lower panel: *GFP* expression on CD45$^+$/Sca1$^+$ cells). **(b)** BM-CD45$^+$/Sca1$^+$ cells isolated from injured muscle of mice transplanted with *GFP/Myf5$^{nlacZ/+}$* donor cells expressed *Myf5* in myogenic co-culture, as it was detected using X-gal staining and bright field microsopy. **(c)** BM cells also participated in muscle regeneration *in vivo*, as revealed by GFP immunofluorescence and fluorescence microscopy analysis of muscle sections. **(d–e)** Lethally irradiated C57/BL6-*CD45.1* mice were transplanted with WBM cells isolated from C57/BL6-*GFP-CD45.2* mice. 6 weeks after BM transplantation, mononucleated cells prepared by non-injured (panel e) or injured (panel d) muscles were plated in methylcellulose M3434 (10^5/plate). The colonies were observed at day 8 by fluorescence microscopy.

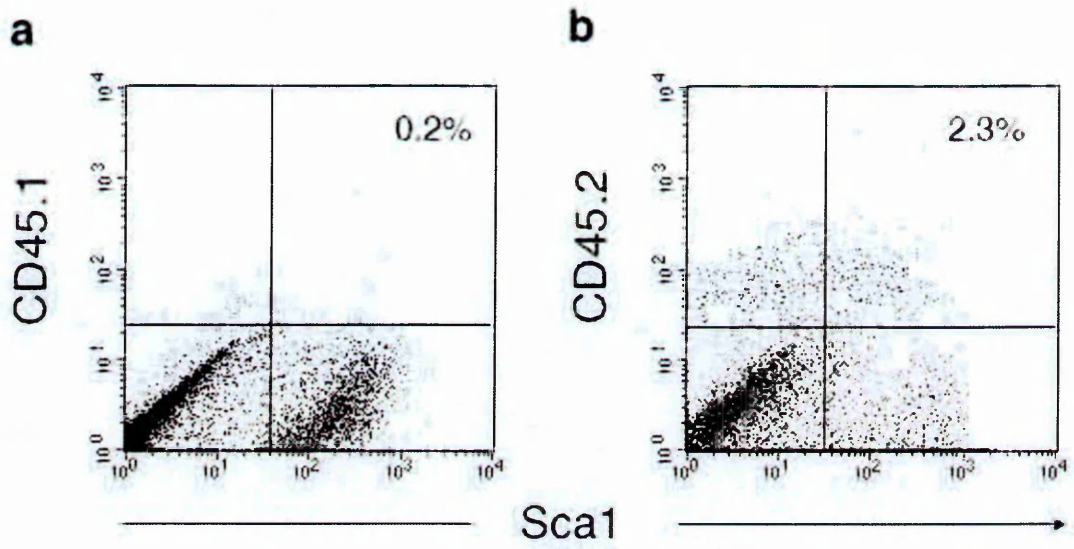

Figure 14. BM cells replenish the muscle-resident CD45+/Sca1+ cells following BM transplantation.

C57/BL6-*CD45.1* mice were lethally irradiated and transplanted with the whole bone marrow cells isolated from C57/BL6-*GFP-CD45.2* mice. Mononucleated muscle cells were prepared from control irradiated (a) or transplanted mice (b) and analysed by flow cytometry for the expression of *CD45.2*, *Sca1* and *CD45.1*, 4 and 21 days after irradiation, respectively.

4.1.3 HSC-derived myogenic progenitors cannot extensively regenerate the muscle.

Our BM transplantation and co-culture experiments clearly demonstrated that particular BM-$CD45^+/Sca^+$ cells are able to migrate to and engraft in skeletal muscle and undergo myogenic specification and differentiation. However, in accordance with several previous reports, the frequency of this process was very low and more importantly these cells never gave rise to myogenic clones in culture. We decided to investigate whether the cause of this infrequency and deficiency, respectively, is the overwhelming myogenic activity of the endogenous muscle stem cells. It is possible that HSC-derived myogenic progenitors need a selective advantage in order to expand and fully exhibit their myogenic potential. To this end, we used $Pax7^{-/-}$ mice as recipients in our BM transplantation experiments, since these mice are characterized by a progressive loss of satellite cells during lifetime and a strong impairement in muscle regeneration. BM cells derived from C57/BL6-*GFP* mice were intravenously injected in lethally irradiated $Pax7^{-/-}$ or $Pax7^{+/-}$ mice, and six weeks after transplantation, ctx injury was used to induce muscle regeneration in the TA muscles. Three weeks later, the animals were sacrificed and GFP immunofluorescence was employed to identify GFP^+ myofibers and to evaluate the contribution of BM cells in muscle regeneration. Interestingly, the level of BM-derived myogenesis was similarly low in both recipients (Figure 15), revealing that BM cells cannot extensively regenerate the muscle, even in a model in which the muscle regeneration by the endogenous cells is highly impaired.

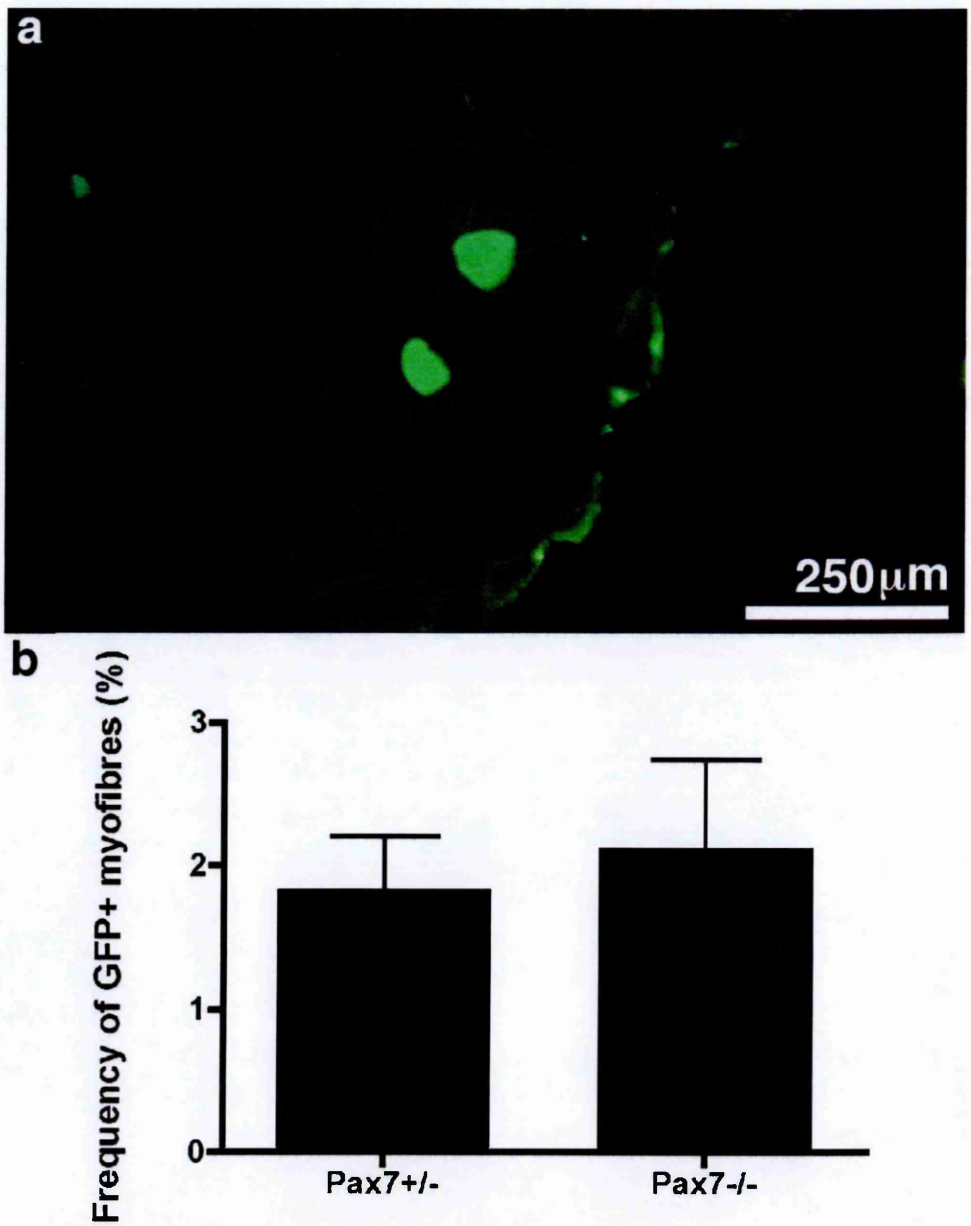

Figure 15. BM cells cannot extensively regenerate the muscle.

Lethally irradiated *Pax7*$^{+/-}$ and *Pax7*$^{-/-}$ mice were transplanted with the whole bone marrow cells isolated from C57BL/6-*GFP* mice. 6 weeks after BM transplantation, cardiotoxin was injected into the TAs and 3 weeks after the injury, immunofluorescence was performed on TA cryosections. **(a)** Fluorescence microscopy of GFP^{+} myofibers identified on muscle sections. **(b)** A bar graph reveals that the frequency of GFP^{+} myofibers is the same in *Pax7*$^{-/-}$ and *Pax7*$^{+/-}$ recipient mice. Error bars represent the standard error of the means of six TA muscles of six different mice per group.

4.1.4 Comparative gene expression profiling of BM- and muscle-derived CD45$^+$/Sca1$^+$ cells reveals partial but extended reprogramming.

To evaluate the extent of myogenic specification of BM-CD45$^+$/Sca1$^+$ cells in muscle at a global level, we performed gene expression profiling for a comparison of CD45$^+$/Sca1$^+$ cells isolated from the bone marrow and the muscle. BM and muscle cells, but most probably of BM origin as suggested by our BM transplantation experiments (Figure 13a and Figure 14a and b), from C57BL/6 mice were sorted for the haematopoietic surface markers CD45 and Sca1 and their total RNA was extracted and used for microarray analysis. Focusing on the expression of myogenic transcription factors in both populations (Table 10), it appears that BM-CD45$^+$/Sca1$^+$ cells do not express any of the transcription factors implicated in muscle regeneration. Ms-CD45$^+$/Sca1$^+$ cells while showing the expression of *Myf5*, thus confirming our reporter assay, do not express other major myogenic transcription factors (e.g. *Myod* and *Pax7*). A wider inspection revealed that several secondary muscle-associated transcription factors (e.g. *Msx1* and *Tcf15*) are expressed in the muscle–derived cell population. We also specifically checked the expression of 99 muscle-associated genes covering different biological categories (see Appendix). Transcription of the muscle-associated genes by ms-CD45$^+$/Sca1$^+$ cells is not limited to transcription factors but it is extended to the entire genome, and this expression pattern was restricted to ms-CD45$^+$/Sca1$^+$ cells (Figure 16). A comparative gene expression analysis of BM- and ms-CD45$^+$/Sca1$^+$ cells revealed that both cell populations express statistically significantly more haematopoietic genes than it would be expected by chance (right-tailed Fisher Exact test: $p=3.19\times10^{-7}$ for BM and $p=3.92\times10^{-6}$ for muscle), but the muscle-associated genes are over-represented only in the muscle-cell population (right-tailed Fisher Exact test:

p=0.00769, Figure 17a and enclosed electronic file). A functional analysis of the genes expressed by the muscle-derived but not the BM–derived $CD45^+/Sca1^+$ cells (see enclosed electronic file) clearly showed that the highest level of differential expression was detected for the over-representation of genes involved in muscle biology (right-tailed Fisher Exact test: $p=4.31x10^{-10}$, Figure 17b). More than 120 genes involved in several muscle processes, such as contraction and migration, were identified. These data suggest that at least a subset of $CD45^+/Sca1^+$ cells undergoes partial but extended reprogramming, once they are found in the muscle, and points out the importance of the microenvironment on the transcriptome of these cells.

Cell Population / Genes	BM-CD45$^+$/Sca1$^+$	Muscle-CD45$^+$/Sca1$^+$
Myf5	-	+
Mrf4	-	-
Myod	-	-
Myogenin	-	-
Pax3	-	-
Pax7	-	-
Mef2a	+	+
Mef2b	-	-
Mef2c	+	+
Mef2d	-	-
Meox1	-	+
Meox2	-	+
Msx1	-	+
Tcf15	-	+
Lbx1h	-	+

Table 10. Gene expression of myogenic transcription factors by CD45$^+$/Sca1$^+$ cells.

CD45$^+$/Sca1$^+$ cells were isolated either from the BM and the muscle of 6 weeks-old C57/BL6 mice. Their total RNA was extracted and used for differential gene expression analysis (Affymetrix GeneChips). The gene expression of several myogenic transcription factors is presented here (plus for expressed or minus for not-expressed).

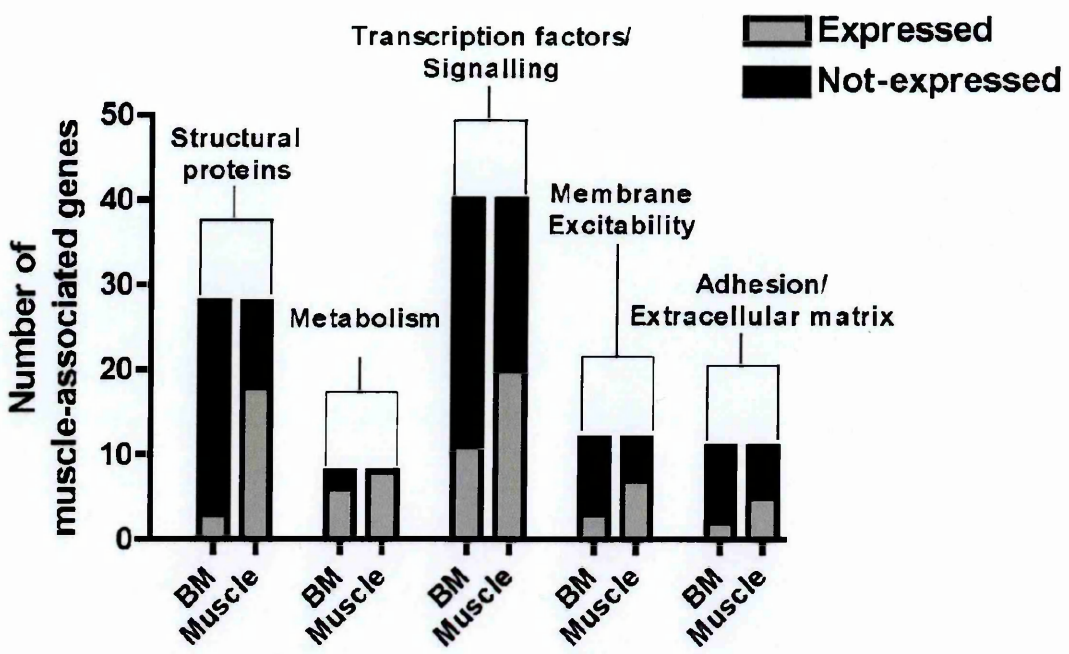

Figure 16. Ms-CD45$^+$/Sca1$^+$ cells express numerous muscle-associated genes.

The expression of 99 muscle-associated genes in the BM- and the muscle-derived cells was checked (expressed and not-expressed are visualised as grey and black bars, respectively). These genes were categorized according to the muscle process in which the corresponding proteins are involved. A bar graph reveals that the expression of the muscle-associated genes by CD45$^+$/Sca1$^+$ cells is extended in all the categories analysed and it is restricted to the muscle-derived population.

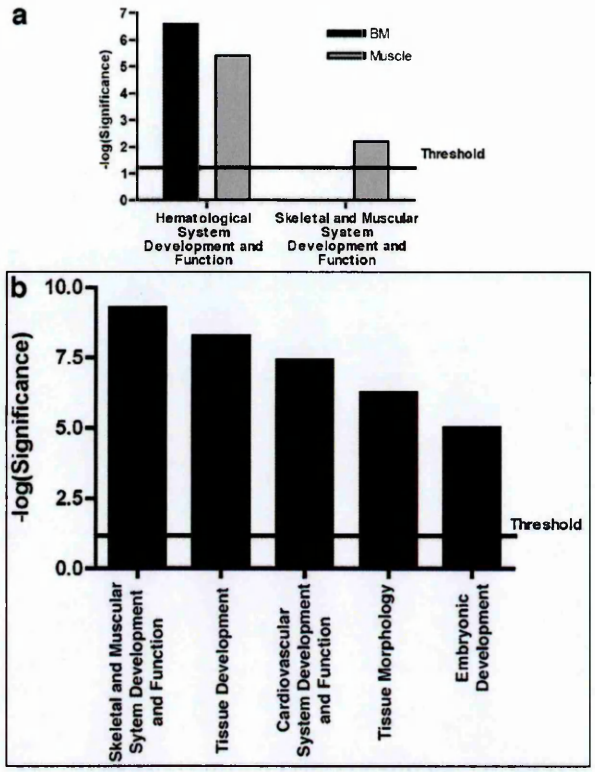

Figure 17. Comparative gene expression analysis of BM- and ms-CD45$^+$/Sca1$^+$ cells reveals differential expression of muscle-associated genes.

(a) The gene expression data of BM- (black bar) and ms- (grey bar) CD45$^+$/Sca1$^+$ cells were compared using the Ingenuity Pathway analysis. A functional analysis specifically focused on haematopoiesis and muscle biology revealed that in both populations there is a statistically significant over-representation of the haematopoietic genes. On the contrary, the muscle genes were statistically significantly over-represented only in the muscle-derived population. **(b)** The functional analysis, of the genes exclusively expressed by the muscle-derived population, identified the biological functions that were most significantly over-represented. The most statistically significant category was "skeletal and muscular system development and function". Here, only the five most statistically significant functions are shown. Bars represent the negative logarithms of the p values, which determine the probability that each assigned function is due to chance alone (right-tailed Fisher Exact test), and the horizontal lines define the statistical threshold (p=0.05).

4.1.5 Pax7 and MyoD are not essential for the myogenic differentiation and participation of HSC-derived myogenic progenitors in muscle regeneration.

Surprisingly, the gene expression profile of $CD45^+/Sca1^+$ cells indicated that *MyoD* and *Pax7* are not transcribed. Due to their central role in muscle development and regeneration (see section 1.1.2.1), this prompted us to investigate their importance in the myogenic potential of HSC-derived myogenic progenitors. In order to use $Pax7^{-/-}$ or $MyoD^{-/-}$ mice as BM donors in BM transplantation experiments and to distinguish their cells from the recipient cells, it was essential to label their HSCs with high efficiency. Therefore, BM cells were infected overnight (MOI: 100) using a lentiviral vector expressing *eGFP* under the human PGK promoter [140]. The transduction efficiency was always higher than 90% for all samples as monitored by flow cytometry (Figure 18), and did not compromise the myogenic potential of haematopoietic cells, since transduced cells efficiently fused to form myotubes in co-culture assay (Figure 19a–d). In addition, to test their *in-vivo* incorporation into myofibers, the transduced BM cells from C57BL/6-*CD45.2* mice were injected into lethally irradiated C57BL/6-*CD45.1* mice. 6 weeks after BM transplantation, muscle regeneration was inducedin the TA muscles using cardiotoxin. We allowed complete muscle regeneration to take place (3-5 weeks) and finally sacrificed the mice and cryosectioned their TAs. Using GFP immunofluorescence, GFP^+ muscle fibres were detected, demonstrating that the transduced haematopoietic cells retain their ability to participate in muscle regeneration (Figure 19e).

We repeated the experiment using $MyoD^{-/-}$ and $Pax7^{-/-}$ (*CD45.2*) mice as donors, and incorporation of the haematopoietic cells into myofibers was measured, counting the GFP^+ muscle fibres. Surprisingly, BM cells lacking MyoD or Pax7 exhibited the

same myogenic potential as cells derived from WT mice (Figure 20a). In order to study further if MyoD and Pax7 are important for the myogenic differentiation of HSC-derived myogenic progenitors, BM cells isolated from WT, *Pax7*$^{-/-}$ and *MyoD*$^{-/-}$ mice were transduced with a virus encoding for eGFP under the control of a chimeric *LTR/MCK* promoter (Cesari *et al*, paper in preparation), with a consistent transduction efficacy greater than 90% for all strains, as measured by PCR detection of the provirus in CFUs (see sections 3.4.14 and 3.4.2). *MCK* is expressed during the differentiation of myoblasts, both before and after the fusion, and this trait allowed us to study both myogenic specification and differentiation. The transduced cells were co-cultured with myoblasts, and several GFP^{+} single cells and myotubes were found (Figure 20b and c), indicating that HSC-derived myogenic progenitors can also express the early differentiation marker *MCK*. More importantly, this expression was not affected by the absence of Pax7 or MyoD (Figure 20d).

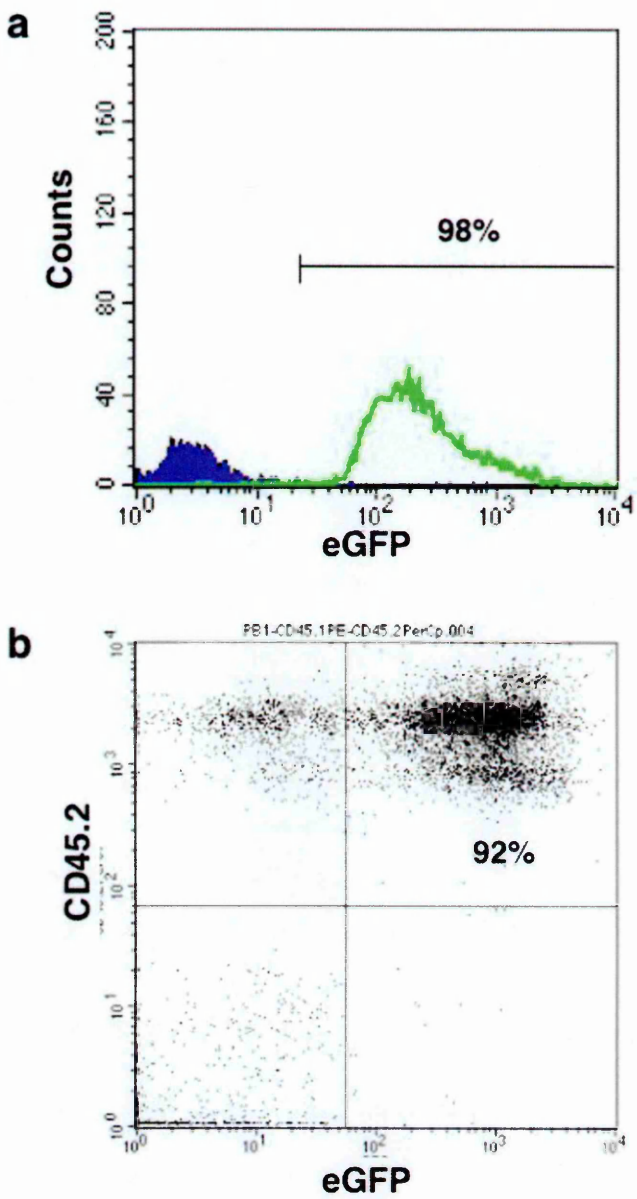

Figure 18. Flow cytometry analysis of transduced BM cells.

BM cells were transduced (MOI 100, O/N) with a vector carrying *eGFP* under the hPGK promoter. **(a)** The transduction efficiency was evaluated 5 days after the infection by flow cytometry analysis. The filled and unfilled histograms represent non-transduced and transduced cells, respectively. **(b)** The transduced BM (*CD45.2*) cells were transplanted into lethally irradiated mice (*CD45.1*). 6 weeks after the BM transplantation, the blood of the recipient mice was analysed by flow cytometry for the expression of *CD45.2* (donor cells) and *eGFP*.

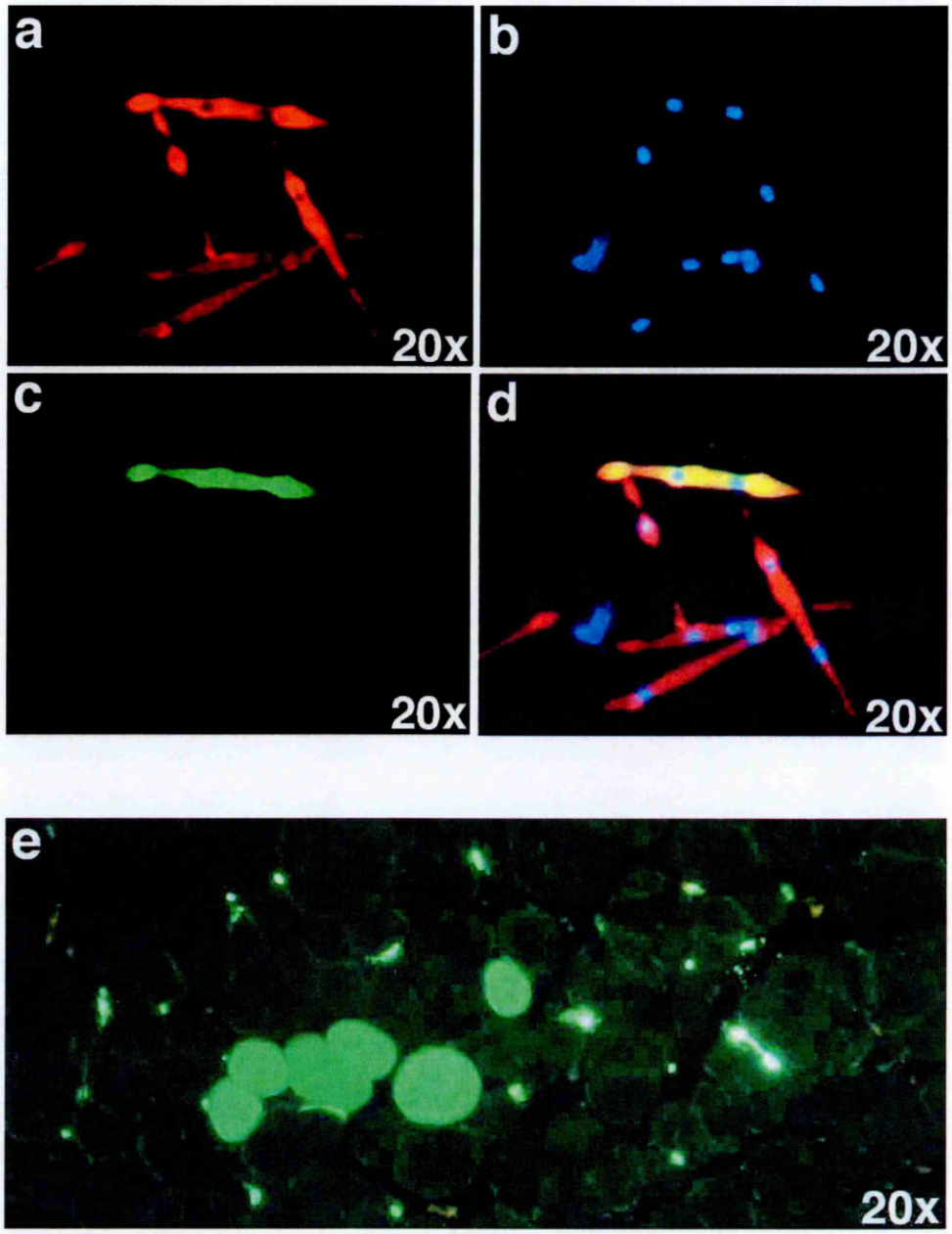

Figure 19. Lentiviral transduction does not impair the myogenic potential of hematopoietic cells.

BM cells were transduced with a vector coding for *eGFP* under the PGK promoter. **(a–d)** Transduced cells co-cultured with myoblasts contributed to myotubes formation as shown by GFP and MHCf immunofluorescence (a: MHCf, b: Hoechst, c: GFP and d: electronic merge). **(e)** Transduced haematopoietic cells were incorporated into TA muscle fibers, following BM transplantation and induction of muscle regeneration (GFP immunofluorescence).

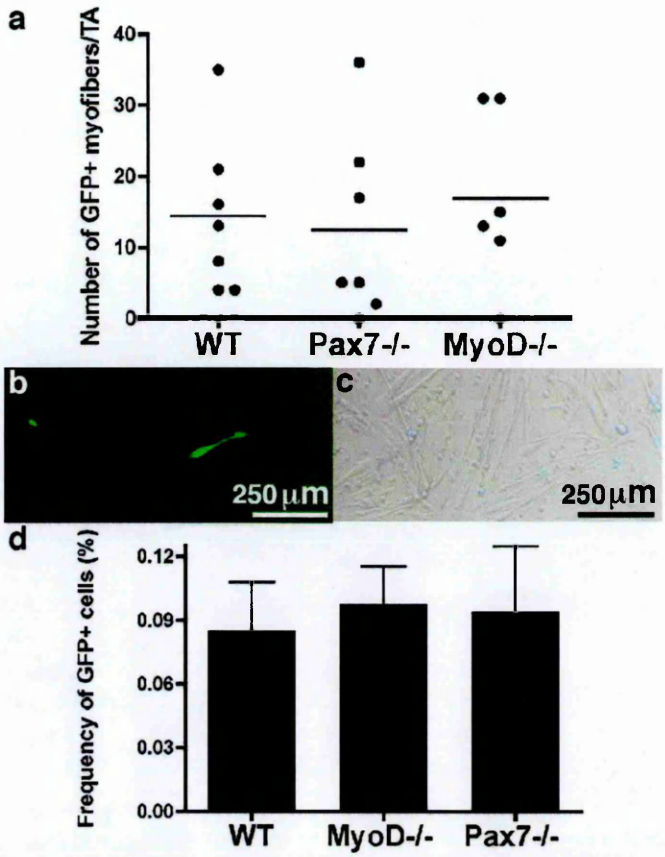

Figure 20. Pax7 and MyoD are not essential for the myogenic differentiation and the participation of HSC-derived myogenic progenitors in muscle regeneration.

(a) BM cells isolated from WT, *MyoD*$^{-/-}$ or *Pax7*$^{-/-}$ mice were transduced with a vector coding for *eGFP* under the PGK promoter and transplanted into lethally irradiated mice. 6 weeks after the BM transplantation, cardiotoxin was injected into the TAs, and 5 weeks after the injury, immunofluorescence was performed on the TA cryosections. Each dot represents one TA of one mouse and the horizontal bars represent the average number of GFP^{+} muscle fibres. **(b–d)** BM cells isolated from WT, *MyoD*$^{-/-}$ or *Pax7*$^{-/-}$ mice were transduced with a vector coding for *eGFP* under the *MCK*/LTR chimaeric promoter and co-cultured with myoblasts. Several GFP^{+} (*MCK*-expressing) cells were observed (b and c: GFP fluorescence and phase contrast microscopy of WT/BM co-cultures, respectively). (d) A bar graph reveals that the BM cells lacking MyoD or Pax7 express *MCK* as frequently as WT cells. The error bars represent the standard errors of the means of six different experiments.

4.1.6 Myogenic specification and differentiation take place in the absence of Pax7.

Pax7-independence initially seemed in contrast with our hypothesis of a step-wise myogenic differentiation of HSC-derived myogenic cells. In addition, in contrast to our findings, Seale and colleagues [45] have claimed that Pax7 is required for the myogenic commitment of the muscle-resident $CD45^+/Sca1^+$ cells. Hence, *Pax7*$^{-/-}$*/Myf5*$^{nlacZ/+}$ and *Pax7*$^{-/-}$/MLC3F-*nlacZ*-E double transgenic mice were bred to ultimately confirm the Pax7-independence of the process. These mice express *LacZ* under the *Myf5* or the *MLC3F* promoter, like *Myf5*$^{nlacZ/+}$ and MLC3F-*nlacZ*-E mice do, but also lack Pax7. Therefore, their utilisation allowed us to monitor the effect of the Pax7 absence on the expression of *Myf5* and *MLC*, respectively. BM cells of these mice were used for myogenic co-cultures, and four days after proliferation (*Pax7*$^{-/-}$*/Myf5*$^{nlacZ/+}$ cells) or 2–3 days after differentiation (*Pax7*$^{-/-}$/MLC3F-*nlacZ*-E cells), several δ-gal^+ cells were detected at frequencies (0.083 ± 0.027 and 0.09 ± 0.015, respectively) not significantly different from the ones observed for BM cells derived from *Myf5*$^{nlacZ/+}$ (0.07 ± 0.025) and MLC3F-*nlacZ*-E (0.096 ± 0.023) mice, which do not lack Pax7 (Figure 21a).

Finally, we decided to investigate if this Pax7-independence of myogenesis is restricted only to HSC-derived myogenic progenitors. Interestingly, the muscle preparations from *Myf5*$^{nlacZ/+}$ and MLC3F-*nlacZ*-E mice in the *Pax7*$^{-/-}$ or the WT background and culture of their muscle cells in myoblast proliferation conditions resulted in cultures dominated by fibroblasts in the case of *Pax7*$^{-/-}$ mice instead of the satellite-like clones observed in WT ones (Figure 21b). However, in the cultures of *Pax7*$^{-/-}$*/Myf5*$^{nlacZ/+}$ and *Pax7*$^{-/-}$/MLC3F-*nlacZ*-E mice, rare cells expressed *Myf5* (at the end of the proliferation) and displayed *MLC* expression and myotubes formation (at the

end of the differentiation), respectively, as revealed by X-gal staining (Figure 21b-f). Notably, most of these cells did not give rise to myoblast clones (Wilcoxon test: $p=0.0014$ for MLC and $p= 0.0312$ for Myf5, Figure 21b and Table 11) and the few myoblast clones detected consisted of a remarkably decreased number of cells (unpaired t test: $p<0.0001$ for MLC and $p<0.0001$ for Myf5, Fig. 7c–f and Table 11). These data indicate that Pax7 is important for the formation and expansion of myoblast clones, and more importantly, demonstrate that myogenic specification (*Myf5* expression) and differentiation (*MLC* expression) are Pax7-independent and this characteristic is shared both by the BM-derived and the muscle-resident myogenic progenitors.

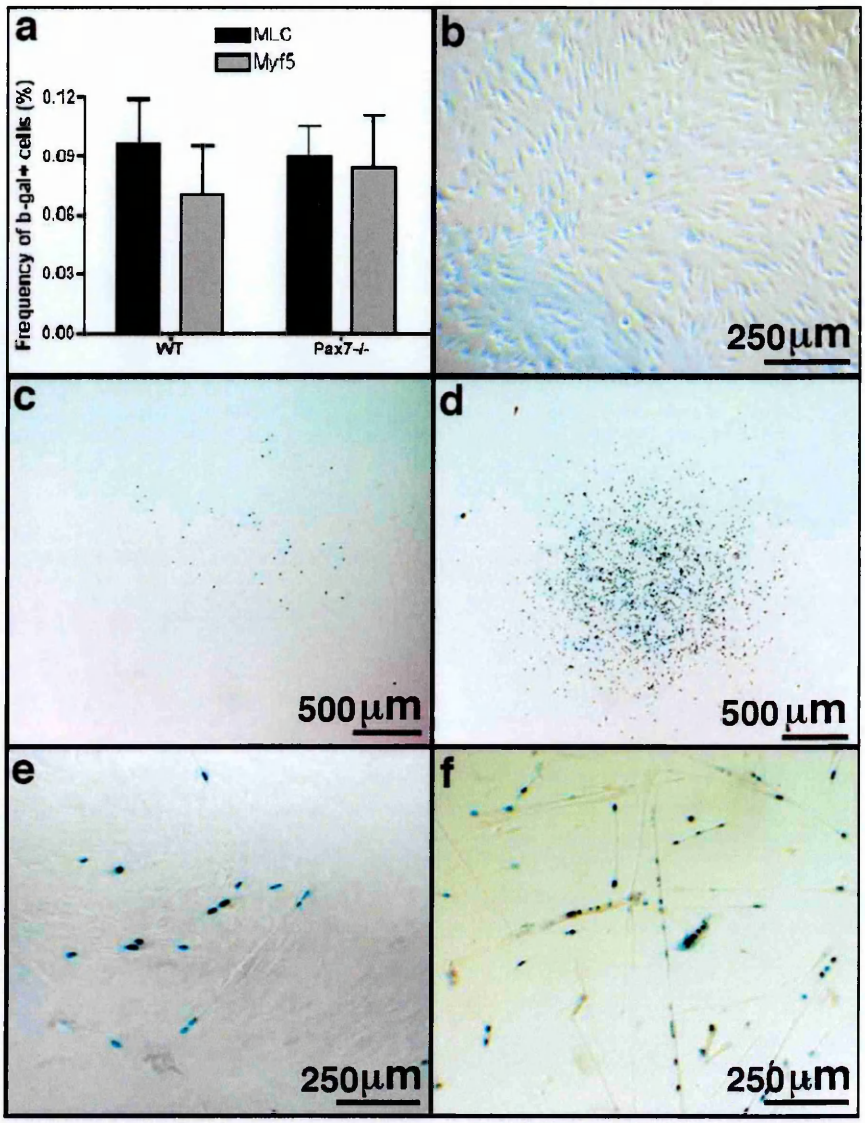

Figure 21. Pax7 absence impairs the formation and expansion of myoblast clones but not the myogenic specification and differentiation.

(a) Myogenic co-culture of the BM cells derived from *Myf5*$^{nlacZ/+}$ and MLC3F-*nlacZ*-E mice in a WT or a Pax7$^{-/-}$ background and subsequent X-gal staining. A bar graph reveals that the BM cells lacking Pax7 express *Myf5* (grey bars) and *MLC* (black bars) as frequently as the WT cells. The error bars represent the standard errors of the means of six independent experiments. **(b–f)** X-gal staining of muscle preparations from the *Myf5*$^{nlacZ/+}$ (proliferation phase) and the MLC3F-*nlacZ*-E (differentiation phase) mice in a WT or a Pax7$^{-/-}$ background (b and c: *Pax7*$^{-/-}$/*Myf5*$^{nlacZ/+}$, d: WT/*Myf5*$^{nlacZ/+}$, e: *Pax7*$^{-/-}$ /MLC3F-*nlacZ*-E and f: WT/MLC3F-*nlacZ*-E).

Mouse genotype	Number of b-gal+ cells ($x10^3$)	Number of myoblast clones	Number of b-gal+ cells/myoblast clone
Pax7$^{-/-}$/*Myf5*$^{nlacZ/+}$	0.5±0.1	6.8±1.2	56±9 (n=59)
WT/*Myf5*$^{nlacZ/+}$	61.2±10.8	124±21	472±31 (n=59)
Pax7$^{-/-}$/MLC3F-*nlacZ*-E	0.2±0.04	2.5±0.4	42±4 (n=107)
WT/MLC3F-*nlacZ*-E	28.5±10.4	82±31	358±17 (n=117)

Table 11. Pax7 absence impairs the formation and expansion of myoblast clones.

The total number of b-gal$^+$ cells, the number of myoblast clones and the number of b-gal$^+$ cells per myoblast clone, as detected by X-gal staining of muscle preparations from the *Myf5*$^{nlacZ/+}$ (proliferation phase) and the MLC3F-*nlacZ*-E (differentiation phase) mice in a WT or a Pax7$^{-/-}$ background. The numbers are mean values and standard errors of the means of six independent experiments, obtained by plating 10^5 total muscle cells.

4.2 Pax7-overexpression activates the intrinsic myogenic potential of BM-CD45$^+$/Sca1$^+$ cells.

The abovementioned data clearly demonstrate that certain BM-CD45$^+$/Sca1$^+$ cells can undergo myogenic specification, differentiation and participation in muscle regeneration, but they never become identical to the satellite cells. Moreover, they confirm that Pax7 is associated with the myoblast-clone forming ability and the satellite-cell stem-cell characteristics, and show that it is not involved in BM-derived myogenesis. We considered the possibility that the inability of HSC-derived myogenic progenitors to behave as muscle stem cells is brought about by this Pax7 absence, and therefore we decided to investigate if the ectopic expression of *Pax7* in these cells would be sufficient for infusing them with the missing Pax7-associated and satellite-like properties and to render them muscle-stem cells or equivalent to satellite cells.

4.2.1 Pax7 up-regulation is sufficient to induce the myogenic specification and the clone-forming ability, but not the differentiation of BM-CD45$^+$/Sca1$^+$ cells.

In order to ectopically express *Pax7*, efficiently and stably, in haematopoietic stem/progenitor cells, we constructed a lentiviral vector, in which the Pax7 cDNA is under the control the human *PGK* promoter (see section 3.4.1). Initially, 293T and HEL cells were infected with the constructed vector, to test the transgene expression. The Pax7 protein was successfully detected in HEL cells, using Western Blot (Figure 22a) and flow cytometry (Figure 22b) and its correct nuclear sub-cellular localisation was shown by immunofluorescence on 293T cells (Figure 22c–f).

BM cells derived from *GFP/Myf5$^{nlacZ/+}$* mice were isolated and transduced with the lentiviral vector, encoding for Pax7 under the control of the *PGK* promoter (MOI:

100, overnight). The infected BM cells were either cultured alone in myoblasts conditions or co-cultured with unlabelled myoblasts and their transduction efficiency was typically 85%, as calculated by flow cytometry (Figure 23d). Importantly, certain *GFP/Myf5*$^{nlacZ/+}$ BM cells over-expressing *Pax7* formed myogenic clones containing 5–20 δ-gal^{+} cells both in culture alone and in myogenic co-cultures (Figure 23a), while the non-transduced ones never did. However, the frequency of the *Myf5*-expressing cells in co-culture was comparable for both cell populations (Figure 23e), due to the rarity of these clones (5 ± 2.5 clones out of 4x10^{5} cells plated, n=3). To ensure that the BM cells capable of this Pax7-activated intrinsic myogenic potential were the CD45^{+}/Sca1^{+} cells under study; we magnetically separated BM-Sca1^{+} cells and therefore CD45^{+}, since all the BM-derived Sca1^{+} cells express *CD45*, from *GFP/Myf5*$^{nlacZ/+}$ mice and we repeated the above co-culture experiment. The enrichment efficiency was higher than 85% (Figure 23b) and as it was expected, BM-CD45^{+}/Sca1^{+} cells generated myogenic clones with a higher frequency than WBM (Figure 23c).

Interestingly, the *GFP*/MLC3F-*nlacZ*-E BM cells, transduced with Pax7, subsequently cultured alone and induced to differentiate, never expressed *MLC*, as revealed by the absence of any δ-gal^{+} cells after X-gal staining (n=6). We considered the possibility that *Pax7* over-expression inhibits the myogenic differentiation of BM-CD45^{+}/Sca1^{+} cells, since it had been reported that Pax7 up-regulation inhibits the differentiation of satellite cells [60]. To exclude this likelihood, we repeated the infection of the *GFP*/MLC3F-*nlacZ*-E BM cells with the Pax7 vector and we co-cultured them with myoblasts. Importantly, the *Pax7* ectopic expression did not abolish the property of BM cells to express *MLC* in co-culture (Figure 23e). Overall, these data demonstrate that the ectopic expression of *Pax7* in BM cells triggers the formation of *Myf5*-expressing myoblast clones, independently from the interaction with myoblasts, but it is not sufficient to induce myogenic differentiation *in vitro*.

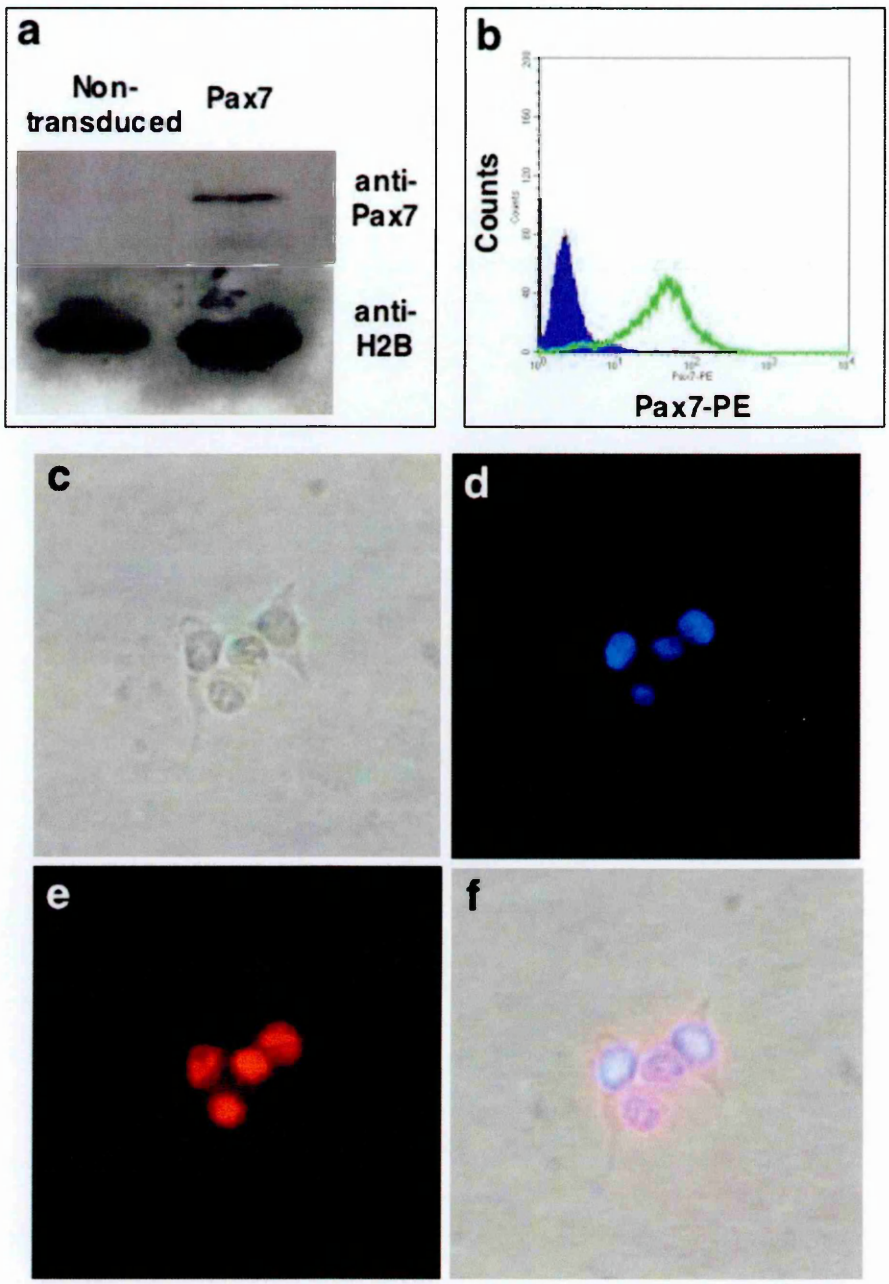

Figure 22. Validation of a lentiviral vector encoding for Pax7.

(a–b) HEL cells were transduced with the Pax7 lentiviral vector and the Pax7 protein was detected both by Western Blot (a, MOI: 1) and flow cytometry (b, MOI: 5). The filled and unfilled histograms represent non-transduced and transduced cells, respectively. **(c–f)** 293T cells were infected with the Pax7 lentiviral vector (MOI: 10, overnight) and the expression of *Pax7* was checked by immunofluorescence microscopy (c: phase contrast, d: Hoechst, e: Pax7 and f: electronic merge).

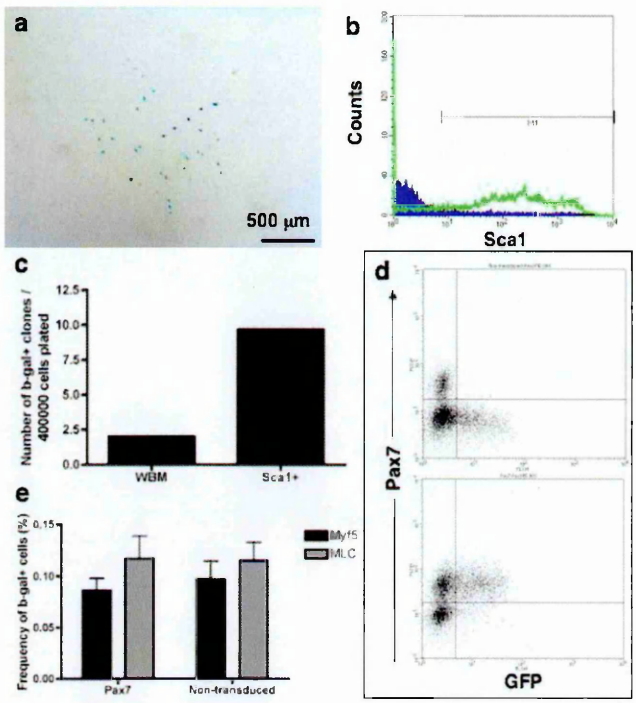

Figure 23. Pax7 up-regulation is sufficient to induce myogenic specification and clones-forming ability, but not differentiation of BM-CD45+/Sca1+ cells *in vitro*.

The BM cells isolated from *GFP/Myf5*$^{nlacZ/+}$ or *GFP*/MLC3F-*nlacZ*-E mice were infected with a lentiviral vector carrying the Pax7 cDNA, **(a–c)** Culture and X-gal staining of the transduced WBM or BM-Sca1^{+} *GFP/Myf5*$^{nlacZ/+}$ cells. (a) A BM-derived *Myf5*-expressing myogenic clone. (b) BM cells magnetically enriched for Sca1 were checked for the expression of the marker by flow cytometry (the filled and unfilled histograms represent WBM and sorted cells, respectively). (c) A bar graph reveals that BM-Sca1^{+} cells created more δ-gal^{+} myoblast clones than WBM. **(d–e)** Co-culture with unlabelled myoblasts and X-gal staining of the transduced BM *GFP/Myf5*$^{nlacZ/+}$ or *GFP*/MLC3F-*nlacZ*-E cells (d): Flow cytometry analysis of *Pax7*- and *GFP*-expression (top panel: non-transduced BM cells and lower panel: transduced BM cells) shows high transduction efficiency. (e) A bar graph reveals that transduced BM cells, ectopically expressing *Pax7*, express *Myf5* (black bars) and *MLC* (grey bars) as frequently as non-transduced ones.

4.2.2 The *Pax7* ectopic expression has no detectable effect on the haematopoietic potential of BM cells.

In vitro, the activation of the intrinsic myogenic program of BM-CD45$^+$/Sca1$^+$ cells, following *Pax7* ectopic expression, encouraged us to investigate this phenomenon *in vivo*. Thus, we decided to use *Pax7*-overexpressing BM cells as donors in BM transplantation experiments; but prior to this, we tested the feasibility of this approach by assessing whether the haematopoietic potential of BM-CD45$^+$/Sca1$^+$ cells is affected by the ectopic expression of *Pax7*. BM cells were either transduced with the *PGK.Pax7* vector or mock-transduced and subsequently plated in methylcellulose media. The frequency of haematopoietic clones was comparable for both populations (Pax7-transduced: 17.8 ± 1.6 CFUs and mock-transduced: 16.5 ± 1.1 out of 2x10^4 cells plated, n=6), suggesting that the *Pax7*-overexpression has no apparent effect on the haematopoietic colony-forming ability of BM cells.

We proceeded with the intravenous injection of *Pax7*-overexpressing or mock-transduced C57BL/6/*GFP/CD45.2* BM cells in lethally irradiated C57BL/6/*CD45.1* mice. The transduction of BM cells, able to repopulate the recipients, was verified by Southern blot and PCR analyses on their genomic DNA, specifically designed for the detection of the integrated provirus (Figure 24). Restriction digestion of the genomic DNA of BM cells isolated from the recipients (3.5 months following transplantation), with an enzyme specifically cutting twice the provirus, allowed us to appreciate the genomic stability of the integrated provirus, since one single band (expected digestion product size: 4500bp) was present in all BM-infected samples. Importantly, Pax7-transduced BM cells reconstituted the haematopoietic system of recipient mice similarly to mock-transduced cells and gave rise to all the haematopoietic lineages tested, as shown by the co-expression of the *CD45.2* donor marker with *B220*, *CD3* or *CD11b* in

peripheral blood (PB) cells analysed with flow cytometry (Figure 25a), three months after transplantation. The level of *Pax7* expression was variable among the mice and the cell lineages and typically ranged between 30%–50%, as revealed by the intranuclear staining for Pax7 and flow cytometry (Figure 25b) .In addition, BM cells isolated from transplanted chimaeras, were used for a clonogenic assay and generated haematopoietic clones with similar efficiencies (Pax7-transduced: 17.5 ± 1 CFUs and mock-transduced: 18.8 ± 0.7 out of $2x10^4$ cells plated, n=6). All the above results, clearly demonstrate that *Pax7* ectopic expression has no detectable effect on the haematopoietic potential of BM cells.

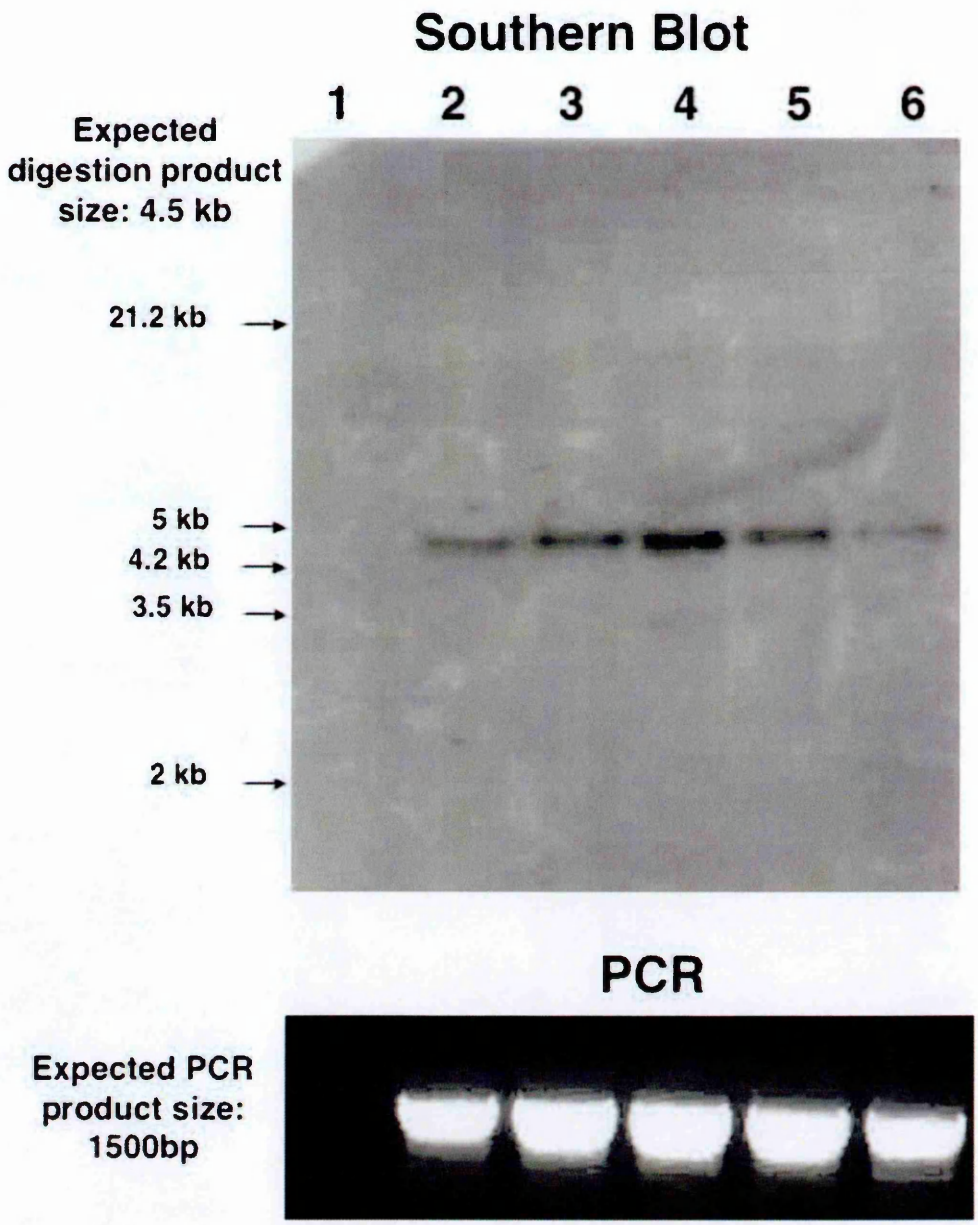

Figure 24. Genomic stability of the integrated *PGK.Pax7* provirus.

BM cells isolated from C57BL/6/*GFP/CD45.2* mice were infected with the *PGK.Pax7* lentiviral vector mock-transduced and transplanted into lethally irradiated C57BL/6/*CD45.1* recipients. After 3.5 months, BM cells from the transplanted mice were isolated for DNA extraction and Southern blot (upper panel) and genomic PCR (lower panel) analyses, to detect the integrated provirus and evaluate its genomic stability (mouse 1: mock-transduced and mice 2–6: transduced).

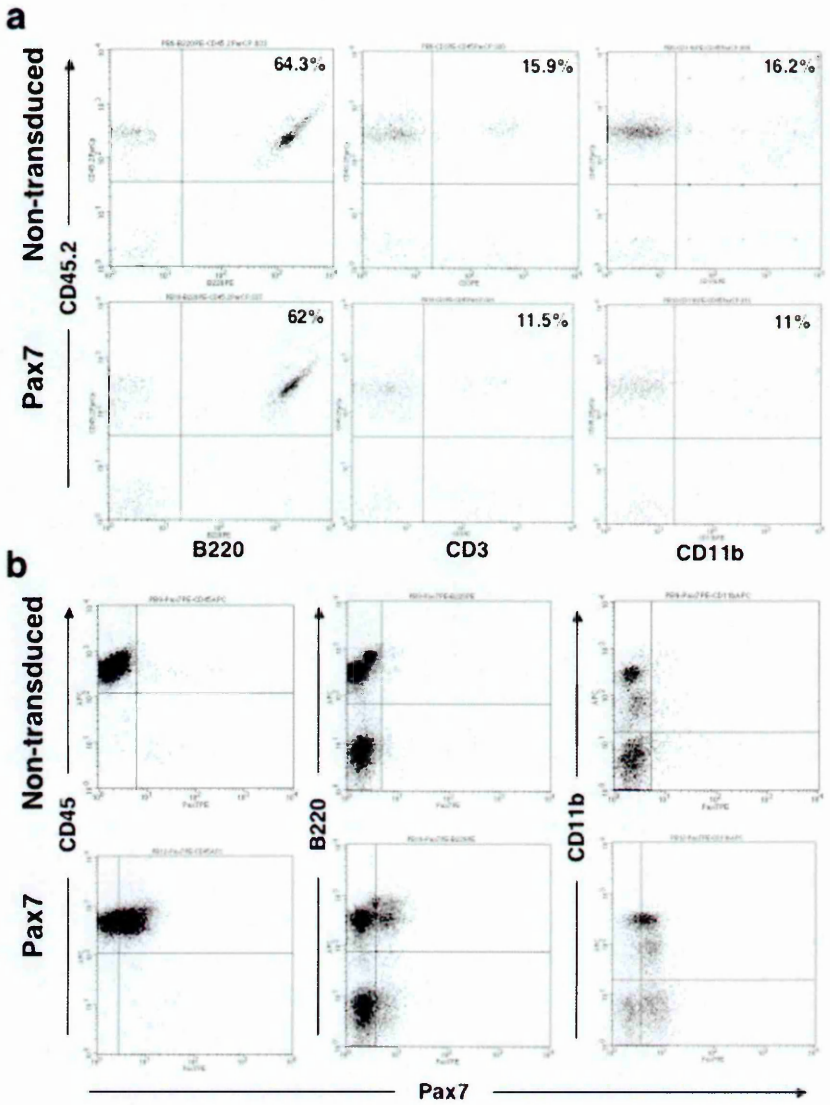

Figure 25. Pax7-tranduced BM cells can reconstitute the haematopoietic system and give rise to all the haematopoietic lineages, *in vivo*.

BM cells isolated from C57BL/6/*GFP/CD45.2* mice were infected with a lentiviral vector carrying the Pax7 cDNA or mock-transduced and transplanted in lethally irradiated C57BL/6/*CD45.1* recipients. **(a)** Flow cytometry analysis of PB for *CD45.2*, *B220* (left panels), *CD3* (middle panels), and *CD11b* (right panels) expression, shows comparable reconstitution of all the three lineages by mock-transduced (top panels) and Pax7-trasduced (lower panels) BM cells. **(b)** Flow cytometry analysis of PB for *Pax7*, *CD45* (left panels), *B220* (middle panels), and *CD11b* (right panels) expression, detects Pax7 in lymphoid and myeloid cell lineages.

4.2.3 *Pax7* over-expression increases BM-derived myogenesis *in-vivo*.

Chimeric mice transplanted with Pax7-transduced *GFP/Myf5*$^{nlacZ/+}$ BM cells were used to assess *in vivo* the level of BM-derived myogenesis, following the *Pax7*-overexpression. Seven weeks after transplantation, the TA muscles of transplanted mice were subjected to ctx injection, to induce muscle regeneration. Seven weeks following muscle injury, the mice were sacrificed and their TAs were cryosectioned and used for GFP immunofluorescence, to quantify the participation of BM cells to muscle regeneration. Notably, we observed a small but statistically significant increase (Mann Whitney test: $p<0.05$) of GFP^+ muscle fibres in the TAs of mice transplanted with the BM cells transduced with Pax7, suggesting that *Pax7* ectopic expression increases their *in-vivo* myogenic potential.

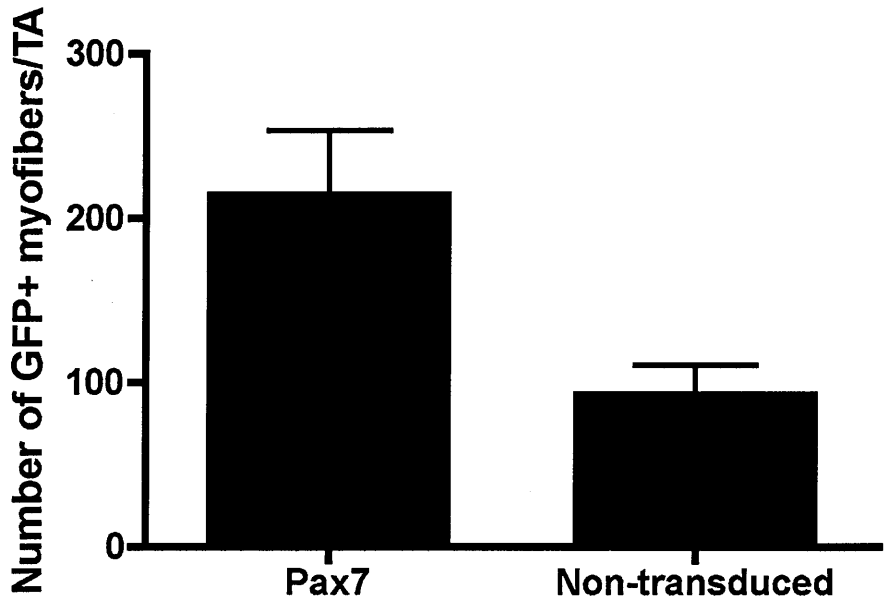

Figure 26. *Pax7* over-expression increases BM-derived myogenesis.

The BM cells isolated from C57BL/6/*GFP/CD45.2* mice were either infected or non-infected with the *PGK.Pax7* lentiviral vector and transplanted into lethally iradiated C57BL/6/*CD45.1* mice. Seven weeks later, cardiotoxin was injected into the TAs of the chimaeras, and 5 weeks after the injury, immunofluorescence was performed on TA cryosections. A bar graph shows that the BM cells transduced with Pax7 incorporate into muscle fibres more readilly than the non-transduced cells. The error bars represent the standard errors of the means of eight different experiments.

5 Discussion

In 1998, our group had demonstrated that BM cells can participate in muscle regeneration [102]. This observation has started an ongoing debate on the myogenic potential of the BM-derived cells, and more generally, on the plasticity of adult stem cells [143]. The terms adult stem cell plasticity and transdifferentiation have acquired controversial and provocative meanings, and in this debate the myogenic potential of BM cells has become a frontline issue, since most studies investigated this phenomenon. Unfortunately, this extensive study has produced controversial data, mainly due to the infrequency of the event [105,106,108,114,127]. The ability of BM cells to activate a myogenic program and to be entirely reprogrammed, and their lineage relationship with the satellite muscle-stem cells remain still uncertain. This research investigated these issues and the data obtained are interpreted in this section.

5.1 Molecular mechanism regulating the BM-derived myogenesis.

The existence of a myogenic program able to regulate the transition of BM cells to muscle cells is surely the most debated aspect of BM-derived myogenesis. It has been reported that BM cells are able to express muscle-specific and satellite-marker genes, like *Myf5*, *c-met*, *CD34*, *M-cadherin* and *Pax7*, prior to their fusion to the muscle fibre both *in vitro* and *in vivo* [105,108,126,127]. However, the reproduction of these results was partial [127] or impossible [114], casting doubts over the integrity of these studies and proposing the idea of direct and accidental fusion of BM cells to the myofiber.

5.1.1 BM-CD45$^+$/Sca1$^+$ cells undergo myogenic specification and differentiation and incorporation into myofibers.

In our experiments, the utilisation of reporter genes under the control of muscle-specific promoters and knock-out mice, and the gene expression profiling of CD45$^+$/Sca1$^+$ cells have enabled a more detailed and comprehensive investigation of the phenomenon. Specifically, co-culture of the *Myf5*$^{nlacZ/+}$ BM-derived cells, enriched for CD45, Sca1, and c-kit, with myoblasts revealed that certain BM-CD45$^+$/Sca1$^+$ cells can express (although infrequently) the satellite marker *Myf5*, before fusion. Furthermore, the utilisation of MLC3F-*nlacZ*-E mice in the co-culture assay has allowed us to detect single cells that express *MLC* and myotubes containing BM-derived nuclei. Similar results were obtained when BM cells, previously transduced with a lentiviral vector encoding for *eGFP* under the control of a chimeric *LTR/MCK* promoter, were used for the co-culture experiments. Interestingly, our BM transplantation experiments revealed that mononuclear BM-CD45$^+$/Sca1$^+$ cells isolated from regenerating muscle also express *Myf5* after co-culture with myoblasts. These results demonstrate that BM-derived haematopoietic stem/progenitor cells can (approximate frequency 0.1%) undergo myogenic specification and differentiation. Notably, our results do not exclude that other BM sub-populations could contribute to the muscle-fibre formation without any previous myogenic pre-specification, as it had been reported previously [106,114,127].

5.1.2 BM-derived myogenesis is MyoD and Pax7 independent.

The gene expression profiling of the CD45$^+$/Sca1$^+$ cells isolated from the muscle ultimately confirmed the expression of *Myf5, MLC* and *MCK*. Nevertheless, *MyoD* and *Pax7* were not expressed, indicating that these two important myogenic transcription factors are not involved in the participation of BM-CD45$^+$/Sca1$^+$ cells in muscle regeneration. Importantly, the experiments of co-culture and BM transplantation, with BM cells from *WT, Pax7$^{-/-}$ or MyoD$^{-/-}$* mice transduced with the MCK.*eGFP* or the PGK.*eGFP* vector, demonstrated that Pax7 and MyoD are not essential for *MCK* expression and contribution of BM-derived haematopoietic cells in muscle regeneration. The MyoD-independence of this process can be explained by its well-established genetic redundancy with Myf5 [144]. Interestingly, developmental studies reported that *MyoD* and *Myf5* are activated in distinct muscle-cell lineages, suggesting that do not compensate for the absence of each other within the same cell but they operate and determine separate muscle-cell lineages [145]. Although, this complementation at cellular level does not seem to be the case for the adult muscle stem cells, since satellite cells express both genes (see section 1.1.2.1), this could explain the solitary *Myf5* expression in HSC-derived myogenic progenitors.

Notably, Pax7 has distinct but also partially overlapping functions with Pax3 both during muscle development [146] and regeneration [44,47], and therefore we considered the possibility that the Pax7-independence observed is conferred by Pax3-activity. However, we excluded this likelihood, since ms-CD45$^+$/Sca1$^+$ cells did not express *Pax3*, as revealed by the DNA microarray data. The Pax7-independence initially appears to contradict our hypothesis of a step-wise myogenic differentiation of haematopoietic cells, owing to the central role that Pax7 has in myoblast proliferation and self-renewal [44-47]. Furthermore, it is opposing with the Pax7-dependent myogenic

specification suggested by Seale and colleagues [78]. On the other hand, Asakura and colleagues [71] have described the Pax7-independence of the myogenic differentiation of ms-SP cells, which also exhibit both haematopoietic and myogenic potential. To undoubtedly clarify the role of Pax7, we performed BM-myoblast co-cultures, utilizing *Pax7*$^{-/-}$/*Myf5*$^{nlacZ/+}$ and *Pax7*$^{-/-}$/MLC3F-*nlacZ*-E mice, and we detected *Pax7*$^{-/-}$ BM-derived haematopoietic cells able to express *Myf5* and *MLC*. In addition, muscle cells derived from these mice also expressed *Myf5* and *MLC*. These findings not only confirm that HSC-derived myogenic progenitors incorporate into the myofibers in a Pax7-independent manner but they also demonstrate that Pax7 is not necessary for myogenic specification and differentiation of both the haematopoietic and the muscle cells.

5.1.3 Indications for alternative myogenic programs.

The acknowledgment of Pax7- and MyoD- independence together with the absence of other important myogenic transcription factors, such as Mrf4, myogenin and Pax3, challenges the step-wise myogenic specification and differentiation observed in our *in-vitro* and *ex-vivo* gene reporter assays. It has been reported that Myf5 fails to support myogenic differentiation in *myogenin*$^{-/-}$/*MyoD*$^{-/-}$/*Mrf4*$^{-/-}$ triple mutant embryos (although it was sufficient to initiate myogenic specification) [147], and therefore it appears difficult to envisage a myogenic program that excludes all the major myogenic transcription factors except for Myf5. However, a more detailed inspection of the DNA microarray data allowed the detection of several secondary muscle-associated genes, like *Msx1* [148] and *Mnf* [149], and transcription factors important for the muscle development and the migration and myogenic specification of the muscle precursor cells (e.g. Meox1 [150], Meox2 [150], Tcf15 [151] and Lbxh1 [152]). It is tempting to speculate that the cooperative expression of these muscle-assosiated genes is sufficient to drive myogenic specification, differentiation and contribution to muscle fibre formation.

It is also probable that the Pax7-lacking cells follow an alternative or partial myogenic program. Recently, it was reported that the ectopic expression of a Pax7 dominant negative mutant affects the expression of MyoD but not Myf5. The same ectopic expression in the *Myf5*$^{+/-}$ satellite cells has no effect on their differentiation, while the transduced *Myf5*$^{-/-}$ satellite cells do not differentiate. These results suggested that either Pax7, acting via MyoD, or Myf5 are required for the myogenic differentiation of satellite cells [44]. The existence of these two parallel genetic pathways explains the Pax7-independence displayed by HSC-derived myogenic progenitors, and taken together with our array data, showing the absence of *Pax7* and *Pax3* expression, suggests that the Pax7 pathway is inactive and only the Myf5 pathway is active in these

cells. It would be interesting to investigate whether the myogenic specification, differentiation and contribution to muscle regeneration of BM-CD45^{+}/Sca1^{+} cells is dependent on Myf5, and therefore whether Myf5 drives BM-derived myogenesis or it is simply one of the genes activated by the muscle microenviroment.

5.2 Nuclear reprogramming of BM-CD45$^+$/Sca1$^+$ cells and muscle microenviroment.

Our reporter gene assays did not reveal the degree of commitment and nuclear re-programming that HSC-derived myogenic cells reach. Fortunately, the DNA microarray analysis permitted us to assess the extent of myogenic reprogramming undertaken by these cells. The analysis of the gene expression profiling of the BM-derived and the muscle-resident CD45$^+$/Sca1$^+$ cells revealed that only the latter cell population expresses statistically significantly more muscle-associated genes than it would be expected by chance. This was verified both by the comparative gene expression analysis between the cell populations and the functional analysis performed on genes exclusively expressed by the ms-CD45$^+$/Sca1$^+$ cells. Considering that the muscle-derived CD45$^+$/Sca1$^+$ cells most probably have a BM origin, as it is strongly suggested by our BM transplantation experiments and previous studies [129,132], the aforementioned differences between the cells isolated from BM and muscle reveal the importance of the microenvironment on the transcriptome and the potential of these cells. This is further supported by the necessity that these cells show for co-culture with myoblasts, in order to differentiate *in vitro*.

In agreement with previous reports (see section 1.2.2), BM-derived CD45$^+$/Sca1$^+$ cells isolated from the muscle retained their haematopoietic potential, as shown both by the statistically significant over-representation of haematopoietic genes (DNA microarray data) and their ability to generate haematopoietic clones. The muscle regeneration, induced by ctx injection, abolished this latter ability of BM-derived CD45$^+$/Sca1$^+$ cells, suggesting a change in their fate. However, it is not possible to associate this change with the acquirement of the myogenic potential, since BM-CD45$^+$/Sca1$^+$ cells exist as a heterogenous population and our experiments cannot

demonstrate that the cells losing their haematopoietic potential obtain myogenic potential and finally participate in muscle regeneration.

Lastly, although our results strongly suggest an extended activation of muscle genes, it is unclear if these cells complete the BM to muscle transition and express the whole repertoire of the muscle genes after their fusion to muscle fibre. Indeed, several studies had reported that the BM cells incorporated into the muscle fibres fail to produce the late muscle-differentiation markers, such as dystrophin [114] or δ-sarcoglycan [109], and proposed models, predicting that different degrees of reprogramming can exist [153] and that a threshold must be reached to achieve the transcription of all the muscle genes [154].

5.3 Relationship between HSC-derived myogenic progenitors and satellite-stem cells.

Previous studies had claimed that BM cells are capable of occupying the sublaminar space, undergoing myogenic specification and giving myoblast-like clones once isolated from the muscle, suggesting that these cells can actually become satellite cells [105,108]. However, this idea has been questioned by numerous reports, addressing their ability to express muscle-specific genes prior to fusion [106,114,127].

In our experiments, $CD45^+/Sca1^+$ cells, isolated directly from the BM or the muscle tissue, did not exhibit the satellite-cell-associated ability to form myoblast clones, demonstrating that they are not identical to satellite cells. The BM transplantations in *Pax7*$^{-/-}$ recipient mice, clearly showed the inability of these cells to extensively regenerate the muscle even in mutant mice in which the satellite-cell population is greatly diminished, and therefore ensure that the always-reported scarce participation of BM cells in muscle regeneration is not due to competition with the endogenous satellite cells. Finally, the comparison of our genomics data on $CD45^+/Sca1^+$ cells with the known properties of satellite-cell-derived myoblasts reveals the partial reprogramming of the former and demonstrates their distinction from the latter. Concerning, the previously claimed localisation of BM cells in the satellite-cell niche [105,108,155], our experiments did not address this feature and consequently we cannot exclude it. However, this characteristic alone is not sufficient to elevate these BM cells to muscle stem cells, since the satellite-cell population is heterogenous and thus not all the cells located in this space are true muscle stem cells. Overall, this study unequivocally establishes that BM-$CD45^+/Sca1^+$ cells cannot be considered muscle stem cells.

5.4 The Pax7 importance on the muscle-stem-cell characteristics.

We previously discussed the Pax7-independence of myogenic specification and differentiation of both muscle cells and BM-derived haematopoietic stem/progenitor cells. However, we ought to underline that the myogenic progenitors derived from the *Pax7*$^{-/-}$ muscles gave rise to very few myogenic clones, containing a decreased number of cells and hence not exhibiting this characteristic Pax7–associated propagation property of satellite cells. Another study in *Pax7*$^{LacZ/LacZ}$ mice revealed that there is an interstitial Pax7-independent myogenic population distinct from the satellite cell compartment [47]. Whether the myogenic progenitors isolated from the muscle of *Pax7*$^{-/-}$ mice is a distinct population from satellite cells or they are satellite cells deprived from their self-maintaining and proliferation ability is not addressed by the current experiments, but it can be studied using the *Pax7*$^{-/-}$/*Myf5*$^{nlacZ/+}$ and the *Pax7*$^{-/-}$/MLC3F-*nlacZ*-E mice.

Notably, the HSC-derived myogenic progenitors, described in our study, never gave rise to myogenic clones nor behaved as muscle-stem cells. Remarkably, activation of the Pax7 pathway in these cells, using the overexpression of *Pax7*, infused to them the ability to generate myoblast clones and increased their *in-vivo* myogenic potential. All the above observations not only confirm that Pax7 is closely linked with the propagation and the self-renewal of satellite cells, but more importantly establish it as the key factor for muscle-stem-cell competence.

6 Conclusive remarks and future perspectives.

To sum up, we propose that BM-derived haematopoietic stem/progenitor cells can undergo myogenic specification, differentiation and participation in muscle regeneration; but they never become identical to the satellite cells, as previously reported. Moreover, this process is MyoD- and Pax7-independent and is also shared by certain muscle-resident myogenic progenitors. Our findings do not provide evidence of adult stem cell plasticity or transdifferentiation of the cells under study as these are very strictly defined [143], since most of the criteria (i.e. total nuclear reprogramming, cell commitment) are not fulfilled. However, we show the myogenic reprogramming of $CD45^{+}/Sca1^{+}$ cells by expression profiling and their step-wise myogenic specification, leading to contribution to the myofiber formation. Our study demonstrates that these cells can change fate and show a degree of plasticity, in the broader sense of the term.

The first reports of BM-derived myogenesis created hope for the treatment of muscle diseases, since BM cells are readily accessed, compared to the muscle-resident stem cells, and they are already used in medicine for the treatment of blood disorders. Unfortunately, these expectations were not encountered and the initial enthusiasm was followed by a general depreciation. Nevertheless, even though it is crystal clear that there will be no current or near-future clinical application of these cells, we believe that studying and deeply understanding the basic biology of BM-derived myogenesis is still relevant to the forthcoming cell therapy. Additionally, the investigation of the reprogramming of these BM cells could give useful insights in stem-cell biology and cell-fate determination, and thus we support the continuance of this research.

Specifically, it would be interesting to further examine the role of Myf5 in the myogenic reprogramming of BM-derived haematopoietic stem/progenitor cells and to this end we are currently performing BM transplantation experiments, using Myf5

mutant mice as donors. Impairment in the participation of the Myf5$^{-/-}$ BM cells in the muscle regeneration would unequivocally confirm that an alternative myogenic program regulates BM-derived myogenesis. The effect of *Pax7*-overexpression on the muscle-stem-cell characteristics of HSC-derived myogenic progenitors is also of particular interest. Whether the BM cells ectopically expressing *Pax7* only partially gain satellite-cell properties or they become identical to them is not elucidated by our experiments. The gene expression profiling of these cells would facilitate us to definitely appreciate the true outcome of Pax7-transduction and more generally evaluate the result of such program manipulations in the stem-cell fate.

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Abbreviations

AGM:	Aorta-Gonad-Mesonephros
bHLH:	basic Helix-Loop-Helix
BM:	Bone Marrow
CFU:	Colony Forming Unit
CLP:	Common Lymphoid Progenitors
CMP:	Common Myeloid Progenitors
CNS:	Central Nervous System
Ctx:	Cardiotoxin
DA:	Dorsal Aorta
E:	Embryonic Day
ES:	Embryonic Stem cells
GMP:	Myelomonocytic Progenitors
HEL:	Human erythroleukemic
Hgf:	hepatocyte growth factor
HSC:	Haematopoietic Stem cell
Igf-1:	Insulin-like Growth Factor-1
LT-HSC:	Long-Term Haematopoietic Stem cell
MAPS:	Multipotent Adult Progenitor Cells
MCK:	Muscle Creatine Kinase
MEP:	Megakaryotic/Erythroid Progenitors
MHC:	Myosin Heavy Chain
MLC:	Myosin Light Chain
MOI:	Multiplicity of infection
MPP:	Multipotent Progenitors

Mrf:	Myogenic Regulatoty Factor
Mrf4:	Myogenic regulatoty factor 4
Ms- $CD45^+/Sca1^+$ cells	Muscle-derived $CD45^+/Sca1^+$
Ms-SP cells:	Muscle-derived SP cells
MyoD:	Myogenic Differentiation-1 gene
Myf5:	Myogenic factor 5
PAS:	Paraaortic Splanchnopleura
Pax3:	Paired box gene 3
Pax7:	Paired box gene 7
PB	Peripheral Blood
Pro-B:	B Progenitors
qPCR:	quantitative real-time polymerase chain reaction
S:	Somite
Sca1:	Stem cell antigen-1
SDF-1:	Stromal Derived Factor-1
SP:	Side Population
ST-HSC:	Short-Term Haematopoietic Stem cell
TA:	Tibialis Anterior
TNFδ:	Tumor Necrosis Factor δ
TU:	Transducing Unit
YS:	Yolk Sac
WBM:	whole bone marrow

Appendix

Genesymbol	Biological Category
Acta1	Structural proteins
Actc1	Structural proteins
Actn2	Structural proteins
Actn3	Structural proteins
Ak1	Metabolism
Arx	Transcription factors/Signalling
Atp2a1	excitability
Atp2a2	excitability
Bgn	adhesion/Extracellular Matrix Proteins
Casq1	excitability
Casq2	excitability
Cd34	adhesion/Extracellular Matrix Proteins
Cdh11	adhesion/Extracellular Matrix Proteins
Cdh15	adhesion/Extracellular Matrix Proteins
Cdh2	adhesion/Extracellular Matrix Proteins
Cckn1c	Transcription factors/Signalling
Chrna1	excitability
Chrnb1	excitability
Chrnd	excitability
Chrne	excitability
Chrng	excitability
Ckm	Metabolism
Cryab	Transcription factors/Signalling
Dach2	adhesion/Extracellular Matrix Proteins
Des	Structural proteins
Dmd	adhesion/Extracellular Matrix Proteins
Eno3	Metabolism
Eya2	Transcription factors/Signalling
Eya4	Transcription factors/Signalling

Foxc2	Transcription factors/Signalling
Foxk1	Transcription factors/Signalling
Foxo1	Transcription factors/Signalling
Fst	Transcription factors/Signalling
Ifrd1	Transcription factors/Signalling
Itga7	adhesion/Extracellular Matrix Proteins
Lbx1h	Transcription factors/Signalling
Ldh1	Metabolism
Ldh2	Metabolism
Lmna	Transcription factors/Signalling
Mb	Metabolism
Mef2a	Transcription factors/Signalling
Mef2b	Transcription factors/Signalling
Mef2c	Transcription factors/Signalling
Mef2d	Transcription factors/Signalling
Meox1	Transcription factors/Signalling
Meox2	Transcription factors/Signalling
Met	Transcription factors/Signalling
Msx1	Transcription factors/Signalling
Musk	excitability
Myf5	Transcription factors/Signalling
Myf6	Transcription factors/Signalling
Myh1	Structural proteins
Myh2	Structural proteins
Myh3	Structural proteins
Myh4	Structural proteins
Myh6	Structural proteins
Myl1	Structural proteins
Myl2	Structural proteins
Myl3	Structural proteins
Myl4	Structural proteins
Mylpf	Structural proteins

Myod1	Transcription factors/Signalling
Myog	Transcription factors/Signalling
Ncam1	adhesion/Extracellular Matrix Proteins
Nfatc1	Transcription factors/Signalling
Nfatc2	Transcription factors/Signalling
Nfatc4	Transcription factors/Signalling
Nfic	Transcription factors/Signalling
Nfix	Transcription factors/Signalling
Nog	Transcription factors/Signalling
Pax3	Transcription factors/Signalling
Pax7	Transcription factors/Signalling
Peg3	Transcription factors/Signalling
Pfkm	Metabolism
Pkm2	Metabolism
Pln	excitability
Prkcq	Transcription factors/Signalling
Pva	excitability
Sgca	adhesion/Extracellular Matrix Proteins
Six1	Transcription factors/Signalling
Six4	Transcription factors/Signalling
Tcap	Structural proteins
Tcf15	Transcription factors/Signalling
Tncc	Structural proteins
Tnnc2	Structural proteins
Tnni1	Structural proteins
Tnni2	Structural proteins
Tnni3	Structural proteins
Tnnt1	Structural proteins
Tnnt2	Structural proteins
Tnnt3	Structural proteins
Tpm1	Structural proteins
Tpm2	Structural proteins

Tpm3	Structural proteins
Ttn	Structural proteins
Twist1	Transcription factors/Signalling
Uncx4.1	Transcription factors/Signalling
Vcam1	adhesion/Extracellular Matrix Proteins
Zfp148	Transcription factors/Signalling